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THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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LIBRARY
WOODS HOLE, MASS.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE TREASURER

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

In the Treasurer's Report for 1950 it was stated that because of increasing costs "the margin is much too slim" and "additional income for current operating expenses is still of the utmost urgency." During 1951 this additional income was not forthcoming, the "margin" disappeared completely, and current expenses for the year exceeded current income by approximately $10,000. Total cash receipts, including $50,000 given by the Rockefeller Foundation for special apparatus, in addition to the $20,000 given for current expenses, were $362,293.29. Total expenditures, including $44,596.54 for this special apparatus, amounted to $371,531.82. As a result, cash on hand at the end of the year was only $3,853.96.

The results of the operations of the Mess and the Supply Department were especially disappointing. The Mess operated at a loss of $6,179.43. If depreciation and overhead charges are also deducted, the loss was $10,260.68. The Supply Department, after adjustments for the value of materials furnished to instruction and research, yielded a profit of $332.79. If depreciation is deducted, however, this is changed to a net loss of $3,189.36.

Because of higher prices for common stocks, the market value of the securities in the Endowment Funds increased by $39,862.37 during the year, to a total of $1,108,645.75. The average yield on securities was 4.63% on book value, 4.05% on market value. The investments and percentages in the Endowment Funds as of January 1, 1952 were:

	Book Value	%	Market Value	%
Bonds				
U. S. Government	$369,997.66	38.15%	$ 357,532.00	32.25%
Railroad	54,613.02	5.63	54,017.00	4.87
Public Utility	90,192.50	9.30	84,400.00	7.61
Industrial	34,800.00	3.59	33,110.00	2.99
Total Bonds	549,603.18	56.67	529,059.00	47.72
Preferred Stocks	127,173.50	13.11	115,627.00	10.43
Common Stocks	290,361.93	29.94	461,188.00	41.60
Total Securities	967,138.61		1,105,874.00	
Principal Cash	2,771.75	.28	2,771.75	.25
Totals	$969,910.36	100.00%	$1,108,645.75	100.00%

The Balance Sheet, Statement of Current Surplus, and Summary of Cash Transactions, as given in the report of the auditors, Seamans, Stetson and Tuttle of Boston, follow:

Marine Biological Laboratory Balance Sheet, Dec. 31, 1951

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of the Hanover Bank, New York, Trustee		$ 969,910.36	
Securities and Cash in Minor Funds		19,255.94	$ 989,166.30
Plant Assets:			
Land		$ 113,626.38	
Buildings		1,490,666.08	
Equipment		304,170.63	
Library		418,796.42	
		$2,327,259.51	
Less Reserve for Depreciation		817,862.69	$1,509,396.82
Current Assets:			
Cash		$ 13,340.77	
Mortgage Note Receivable		2,350.00	
Accounts Receivable		18,637.27	
Inventories:			
Supply Department	$ 54,719.00		
"Biological Bulletin"	16,467.20	$ 71,186.20	
Investments:			
Devil's Lane Property	35,347.55		
Stock in General Biological Supply House	12,700.00		
Other Investment Securities	46,101.25		
Retirement Fund	21,740.46	115,889.26	
Prepaid Insurance		6,406.32	
Items in Suspense (Debits)		1,716.87	$ 229,526.69
			$2,728,089.81

Liabilities

Endowment Funds:			
Endowment Funds	$ 967,821.66		
Reserve for Amortization	2,088.70	$ 969,910.36	
Minor Funds		19,255.94	$ 989,166.30
Plant Funds:			
Mortgage Notes Payable		$ 11,000.00	
Donations and Gifts	$1,375,719.68		
Other Investments in Plant from Gifts and Current Funds	122,677.14	1,498,396.82	$1,509,396.82
Current Liabilities and Surplus:			
Accounts Payable		$ 11,319.23	
Items in Suspense (Credits)		1,055.00	
Current Surplus		217,152.46	$ 229,526.69
			$2,728,089.81

Current Surplus Account

Balance, January 1, 1951			$ 231,514.60
Add:			
Excess of Income over Expense for Year		$ 14,245.09	
Reserve for Depreciation, 1951, Charged to Plant Funds		32,181.00	46,426.09
			$ 277,940.69
Deduct:			
Payments during year for Plant Assets:			
Equipment:			
From Current Funds	$ 4,344.66		
From Rockefeller Foundation Grant	44,020.64	$ 48,365.30	
Library:		12,422.93	60,788.23
Balance, December 31, 1951			$ 217,152.46

Summary of Cash Transactions for Year ended December 31, 1951

		Expenditures		
Current Cash	Receipts	Current	Additions to Capital Assets	Total Expenditures
Income from Endowment Funds	$ 44,898.43			
Income from Other Investments	22,517.60			
Donations, Rockefeller Foundation	70,000.00			
Donations, Others	5,240.00			
Real Estate Rentals	7,999.92	$ 1,011.75		$ 1,011.75
Membership Dues	2,442.00			
Instruction	15,170.00	10,229.11		10,229.11
Research (including Apparatus and Chemical Departments)	28,955.00	29,748.17	$ 44,596.54	74,344.71
Supply Department	68,883.66	77,563.91		77,563.91
Mess	45,843.27	54,694.47	1,285.48	55,979.95
Dormitories	22,405.56	7,970.10		7,970.10
"Biological Bulletin"	8,432.28	11,676.82		11,676.82
Library	4,539.26	9,593.12	9,161.99	18,755.11
Dr. Frank R. Lillie Memorial Fund—Arbacia Survey	3,070.03	2,616.28		2,616.28
Miscellaneous (Net)	1,437.74			
Office of Naval Research	1,998.61	2,000.00		2,000.00
Book Purchase Account, Woods Hole Oceanographic Institution	1,200.00	1,100.27		1,100.27
Real Estate Transactions	7,259.93	7,350.00		7,350.00
Administration		26,448.66		26,448.66
Buildings and Grounds		52,368.61	2,483.28	54,851.89
Interest, Insurance and Other Property Maintenance		9,954.46		9,954.46
Payments to Retirement Fund		9,678.80		9,678.80
	$362,293.29	$314,004.53	$ 57,527.29	$371,531.82

Cash Balance, January 1, 1951:		
The Hanover Bank	$ 11,003.27	
Falmouth National Bank	2,089.22	$ 13,092.49
Receipts		362,293.29
		$375,385.78
Payments		371,531.82
Cash Balance, December 31, 1951:		
The Hanover Bank	$ 2,923.71	
Falmouth National Bank	930.25	$ 3,853.96

Other Cash Accounts	Balances Jan. 1, 1951	Received	Paid	Balances Dec. 31, 1951
Allen R. Memhard Fund Cash	$ 83.05	$ 21.10		$ 104.15
Rev. Arsenious Boyer Burse Cash	182.07	116.75		298.82
Lucretia Crocker Fund Cash	674.92	424.91	$ 360.00	739.83
G. H. A. Clowes Fund Cash	1,000.00			1,000.00
Dr. Frank R. Lillie Memorial Fund Cash	8,331.84	2,725.00	3,070.03	7,986.81
Retirement Fund Cash	999.35	10,131.10	8,635.00	2,495.45
Bio Club Scholarship Fund Cash	72.53	54.71		127.24
Book Fund Cash	3,293.48		3,293.48	
Lalor Foundation Fellowships Cash	2,060.93	6,000.00	6,706.51	1,354.42

Respectfully submitted,
Donald M. Brodie,
Treasurer

V. REPORT OF THE LIBRARIAN

The appropriation for 1951 Library expenses was $14,255.68, plus $4500 from the Woods Hole Oceanographic Institution to be applied to staff salaries. This sum was expended as follows: Serials, $5472.90; Books, $424.50; Back Sets, $698.40; Binding, $2565.68; Supplies and Sundries, $443.19; Freight and Express, $105.13; Insurance, $45.00; Salaries, $9046.00. The total expenditures totalled $18,800.80.

In addition to the above, $1200 (plus a balance of $59.10 from 1950) was received from the Woods Hole Oceanographic Institution for acquisitions covering the field of oceanography. Of this amount, $1127.02 was spent, leaving a balance of $132.08 on Dec. 31, 1951.

The number of current journals received in 1951 remains the same as that for 1950, the new titles offsetting those that have ceased publication. There were 454 (11 new) Marine Biological Laboratory subscriptions; 587 (22 new) exchanges and 161 (14 new) gifts; 72 (7 new) were Woods Hole Oceanographic Institution subscriptions; 173 (13 new) were exchanges and 30 (6 new) were gifts; making a total of 1477 (73 new) current periodicals.

The Marine Biological Laboratory purchased 53 books, received 12 complimentary copies from authors, 45 titles from publishers, and 13 miscellaneous donations. The Woods Hole Oceanographic Institution purchased 26 titles, and received 10 gifts. The total number of new books placed on the shelves was 159.

During 1951, the balance of the Carnegie Corporation of New York Fund was expended. The final report, covering the years 1942–1951, shows that the $25,000 was spent for the following: 92 completed back sets; 111 partially completed back sets; 544 volumes bound; and 52 books (classics). This grant has enabled the Library to lower the number of sets needed. In addition, 29 sets were made nearer complete, most of these being war volumes which had been difficult to secure. The Woods Hole Oceanographic Institution acquired 9 back sets.

The reprint collection was increased by 4714 papers, 1604 being of current issue and the remaining ones of earlier date.

Forty-five volumes were borrowed on inter-library loan and 86 were sent to out-of-town institutions. Seventy-eight microfilm orders were filled, amounting to $141.82.

The amount of $1207.84 was received from the sale of accumulated duplicate journal sets and reprints. A portion of this sum was used very advantageously towards the purchase of a complete set of the Berlin Academy's valuable publications.

The Library had the great fortune to receive the reprint collection belonging to the late Dr. Otto Glaser. In sorting out these papers, many valuable ones were found not to appear in the Library. The bequest on the part of Dr. Glaser symbolized his ever-ready willingness to be of assistance to the Staff and his great interest in the growth of the Library.

Grateful acknowledgment is made to Dr. Roberts Rugh and to Dr. Mary E. Collett for their gifts. Several valuable books were used to replace badly worn copies. The gifts of reprints, including Dr. Glaser's, totalled 11,437, of which 1550 were not already in the collection.

At the end of the year 1951, the Library contained 61,068 bound volumes and 178,795 reprints.

The Librarian wishes to express her appreciation for the willing help and valuable advice received from the Library Committee throughout the year.

Respectfully submitted,
Deborah L. Harlow,
Librarian

VI. REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

I submit herewith a report of the sixty-fourth session of the Marine Biological Laboratory for the year 1951.

During the past year important progress has been made in certain directions including scientific instrumentation, plant improvements, fellowship programs, course developments and collecting facilities. However, the Laboratory will be faced in the near future by serious financial problems which can be adequately met only with carefully planned aggressive action.

1. *Scientific Equipment*

The research possibilities of the Laboratory have been greatly enhanced by the acquisition of important and costly research equipment through a grant generously provided by the Rockefeller Foundation. Mr. Chester I. Barnard, then President of the Rockefeller Foundation, and Dr. Warren Weaver, Director of the Natural Sciences Division, became fully cognizant of the apparatus needs of the Laboratory as a result of their visit during the summer of 1950. The Laboratory applied for and received a grant of $75,000 which has made possible the acquisition of an ultracentrifuge, preparation centrifuge, freeze-drying apparatus, refrigerated centrifuge, Warburg microrespirometers, spectrophotometers, ice-making machine, electronics apparatus, emergency generator and other equipment. The acquisition of this equipment is fully justified by its almost continuous use in research and teaching. As of next year there will be added to our equipment an electron microscope acquired under the same grant.

2. *Plant Improvements*

Continued progress was made during the past year in putting our plant in first class condition. Extensive exterior repairs were required by some of the wooden dormitory buildings. When these have been completed, work will then be pushed on the interiors which require considerable repair.

3. *Instruction*

The course in Marine Ecology will be inaugurated in the summer of 1952 under the direction of Dr. Bostwick H. Ketchum. The establishment of this course has been made possible through a grant of $4000 from the New York Zoological Society. It is hoped that this course will help to broaden the base of research interest at the Laboratory.

Dr. Daniel Mazia was appointed to succeed Dr. E. S. Guzman Barron in charge of the course in Physiology.

4. *Fellowships*

In addition to the Lalor Fellowships which have been so successful over the past several years, there is now available a new fellowship in Neurophysiology, established by the Grass Trust. There were two Grass Fellows this past season. The excellent results achieved in this first year are most promising for a continuation of this fellowship.

Through the generosity of Mrs. Frank R. Lillie, there has been established the Frank R. Lillie Educational Fund which will support a fellowship and provide research space at the Laboratory, starting in the summer of 1952. The fellowship provisions permit the selection of a fellow in Embryology or the broad field of general zoology.

5. *Contributions and Gifts*

The contribution of the Associates of the Laboratory totaled $1920. It was initially planned with this fund to have our boat carpenters build a boat to replace the Sagitta. However, the cost of construction frames proved prohibitive. As the result of a careful survey, it was found that the Sagitta could be put in first class condition by replacing the ribs, certain of the planking, most of the hardware and part of the deck. This has been achieved most successfully. Mrs. Murray Crane made a special contribution of $300 to help cover the costs of this remodeling. Mrs. Edward B. Meigs contributed $500 in memory of her husband, Dr. Edward Browning Meigs, who was a generous friend of the Laboratory and an active investigator.

Mr. and Mrs. George F. Jewett contributed $100 for general Laboratory purposes.

Dr. W. D. Curtis showed his continued interest in the Supply Department by the gift of an outboard motor to provide tender service for the Dolphin on class trips.

These added special gifts from these Associates are all most gratefully acknowledged.

The Kettering Foundation contributed $2500 to the Lillie Memorial Fund. This fund has proved invaluable for the development of special marine collecting facilities. During the past few years there has been a striking interest in a diversity of animal and botanical forms which has required the development of special collecting and transporting methods.

6. *Government Contracts*

The Laboratory has a contract with the Office of Naval Research which provides funds and facilities for the collecting and transport of squid from distant points to the Laboratory. The methods that have been developed have proved most effective and require refrigerated, aerated transport tanks.

A contract is being negotiated with the Atomic Energy Commission to obtain support of the radioisotopes research. It is hoped that research assistants, apparatus and isotopes may be obtained through such a contract.

7. *Bar Neck Property*

There was some prospect that the Navy might acquire the Bar Neck Property for a Navy Oceanographic building. There was strong opposition to this from the Trustees of the Marine Biological Laboratory who were strongly opposed to parting with this very important Laboratory asset, since it did not appear that this site was essential for the proper operation of the projected laboratory. No final solution of this problem has been worked out but it is probable that the appropriation for the land and building is hardly sufficient to permit the acquisition of such a valuable piece of property as the building site.

8. *Grants*

The Laboratory still continues to receive support from the Rockefeller Foundation on a grant made three years ago. This grant has two more years to run with $35,000 available for these two years. This grant has been used for the general running expenses of the Laboratory. Its termination will result in a serious reduction in the funds for these general expenses. Plans must be developed this year to offset this loss in income resulting from the termination of this grant. In fact, it is essential that even more than this be done, that additional funds be found over and above those which have been so generously provided by the Rockefeller Foundation. The wage level for the full-time staff of the Laboratory has not kept pace with the increased cost of living. Also, with an increased income, many improvements can be made in the services rendered to investigators, increasing the effectiveness of their work. The housing situation is also susceptible of considerable improvement.

These problems should receive careful consideration by the Trustees and plans developed which will show real promise of solving the difficulties.

Respectfully submitted,
Phillip B. Armstrong,
Director

1. Memorial

Walter Eugene Garrey

By

F. P. Knowlton

Walter Eugene Garrey was born in Reedsville, Wisconsin, April 7, 1873, the elder son of a pioneer Wisconsin physician, John Eugene and Harriet Anderson Garrey. After receiving his elementary education in the public schools of Wisconsin, he entered Lawrence University from which he was graduated with the degree of Bachelor of Science in 1894. From 1894 to 1898 he was an extension instructor in the schools of Chicago. The year 1898 was spent as a student at the University of Berlin, following which he was appointed Fellow in Physiology in the department of Professor Jacques Loeb at the University of Chicago. There, in 1900, he received the degree of Doctor of Physiology and Pharmacology at Cooper Medical College at San Francisco. During these years at San Francisco he came east each summer to study medicine at Chicago, finally receiving the M.D. degree from Rush Medical College in 1909.

From 1910 to 1916 he was Associate Professor of Physiology in Washington University at St. Louis. In 1916 he was appointed Professor of Physiology at Tulane University where he remained until appointed to the chair of Physiology in the reorganized medical school of Vanderbilt University at Nashville. He served as Professor of Physiology and Chairman of the department at Vanderbilt from 1925 until 1944 when he was appointed Emeritus Professor.

The first course in Physiology at the Marine Biological Laboratory was given by Professor Jacques Loeb in 1899. As Fellow in Physiology, Garrey assisted in the collection and preparation of material for the course. From that time on, excepting only the years he was in San Francisco, his summers regularly were spent at the Woods Hole laboratory. He was an instructor in the course in Physiology until 1925, and continued as research worker and consultant in Physiology until the year of his death. In 1920 the Corporation elected him a member of the Board of Trustees. He served as trustee until 1944 when he became Emeritus Trustee.

Of some 60 papers on which his name appears as author or co-author, nearly half relate to experiments carried out, in whole or in part, in this Laboratory. A fundamental interest in general physiology resulting from his early association with Professor Loeb appears in many of these papers. The physiology of the heart was his major interest throughout most of his life. His experiments on cardiac fibrillation, and a theory of fibrillation based on these experiments, will be remembered as an outstanding contribution to medical science.

Membership on the National Research Council, the National Board of Medical Examiners, and the Council on Physical Therapy of the American Medical Association give evidence of the high esteem in which he was held among scientific workers in diverse fields. The highest possible tribute from colleagues in his own special field came when he was chosen president of the American Physiological Society in 1937 and 1938.

An athlete in his younger days, he remained tall and erect. With his head of white hair he was a noteworthy figure in any gathering. A somewhat gruff exterior covered a friendly and sympathetic personality. As Consultant in Physiology at the Laboratory, his advice and assistance were always freely available to younger workers. A number of papers from this Laboratory which do not bear his name nevertheless owe their origin to suggestions and advice from Dr. Garrey. He was fond of entertainment and of entertaining, and the fact that Mrs. Garrey was a charming hostess made their home a frequent gathering-point for friends and neighbors. Though illness handicapped him in his last years he retained his interest in scientific work; and only last autumn as he journeyed back to Nashville he was outlining work that he hoped to complete. However, further illness intervened and he died at Nashville, June 15, 1951.

The Corporation and members of the Laboratory record the passing of one of those individuals whose broad interests and noteworthy accomplishments have helped to make the reputation of the Laboratory international. Those of us who knew him mourn the loss of an honored friend.

2. The Staff, 1951

Philip B. Armstrong, Director, State University of New York, School of Medicine, Syracuse.

Senior Staff of Investigation

E. G. Conklin, Professor of Zoology, *Emeritus,* Princeton University.
R. S. Lillie, Professor of General Physiology, *Emeritus,* The University of Chicago.
A. P. Mathews, Professor of Biochemistry, *Emeritus,* University of Cincinnati.
G. H. Parker, Professor of Zoology, *Emeritus,* Harvard University.

ZOOLOGY

I. Consultants

F. A. Brown, Jr., Professor of Zoology, Northwestern University.
Libbie H. Hyman, American Museum of Natural History.
A. C. Redfield, Woods Hole Oceanographic Institution.

II. Instructors

L. H. Kleinholz, Associate Professor of Biology, Reed College, in charge of course.
George M. Moore, Professor of Zoology, University of New Hampshire.
C. G. Goodchild, Professor of Biology, S. W. Missouri State College.
John H. Lochhead, Associate Professor of Zoology, University of Vermont.
Madelene E. Pierce, Associate Professor of Zoology, Vassar College.
Ralph I. Smith, Assistant Professor of Zoology, University of California.
T. H. Waterman, Assistant Professor in Biology, Yale University.
Marion H. Pettibone, Arctic Research Laboratory, U. S. National Museum.

III. Laboratory Assistants

Robert S. Howard, Northwestern University.
Muriel Sandeen, Duke University.

EMBRYOLOGY

I. Instructors

S. Meryl Rose, Associate Professor of Zoology, University of Illinois, in charge of course.

Charles B. Metz, Assistant Professor of Zoology, Yale University.
John T. Bonner, Assistant Professor of Biology, Princeton University.
Mac V. Edds, Jr., Assistant Professor of Biology, Brown University.
John R. Shaver, Assistant Professor of Zoology, University of Missouri.
Edgar Zwilling, Associate Professor of Genetics, University of Connecticut.

II. Laboratory Assistants

J. Bruce Guyselman, Northwestern University.
Albert E. Murray, Jr., University of Illinois.

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania.
Otto Loewi, Professor of Pharmacology, New York University, School of Medicine.
Arthur K. Parpart, Professor of Biology, Princeton University.
Albert Szent-Gyorgyi, Director, Institute for Muscle Research.

II. Instructors

E. S. Guzman Barron, Associate Professor of Biochemistry, University of Chicago, in charge of course.
Daniel Mazia, Professor of Zoology, University of California.
Irving M. Klotz, Professor of Chemistry, Northwestern University.
Stephen Kuffler, Associate Professor, Johns Hopkins University.
H. Burr Steinbach, Professor of Zoology, University of Minnesota.
George Wald, Professor of Biology, Harvard University.

III. Laboratory Assistant

Andrew Nemeth, Johns Hopkins University School of Medicine.

BOTANY

I. Consultants

Bostwick H. Ketchum, Woods Hole Oceanographic Institution.
Wm. Randolph Taylor, Professor of Botany, University of Michigan.
George F. Pappenfuss, Professor of Botany, University of Michigan.

II. Instructors

Maxwell S. Doty, Assistant Professor of Biology, University of Hawaii, in charge of course.
R. D. Wood, Assistant Professor of Botany, Rhode Island State College.
Francis T. Haxo, Assistant Professor of Biology, Johns Hopkins University.

III. Laboratory Assistant

Julius Silberger, Harvard University.

IV. Collector

Albert J. Bernatowicz, University of Michigan.

V. Lecturers

J. B. Lackey, Medical and Scientific Editor, The Blakiston Company.
Ruth Patrick, Curator of Limnology, Academy of Natural Sciences of Philadelphia.
John Wallace, Academy of Natural Sciences of Philadelphia.

EXPERIMENTAL RADIOLOGY

G. Failla, College of Physicians and Surgeons, Columbia University.
L. Robinson Hyde, Phillips Exeter Academy, Exeter, N. H.

LIBRARY

Mrs. Deborah Lawrence Harlow, Librarian

Mary A. Rohan
Loretta J. Benevides
Joan M. Hoffer

APPARATUS DEPARTMENT

Robert Mills, Manager

James D. Graham
Betty J. Keeler
Seaver R. Harlow

CHEMICAL DEPARTMENT

Gail M. Cavanaugh, Chemical Supplies Manager

SUPPLY DEPARTMENT

James McInnis, Manager
John S. Rankin, Naturalist

Ruth Crowell
Patricia M. Conway

M. B. Gray
A. M. Hilton
W. E. Kahler
G. Lehy
Carl O. Schweidenback
R. O. Lehy
James Whitcomb
H. S. Wagstaff
Robert Perry

GENERAL OFFICE

Homer P. Smith, General Manager
Polly L. Crowell, Office Manager

Mrs. Lila S. Myers
Mrs. Mary M. Carr

GENERAL MAINTENANCE

R. W. Kahler, Superintendent

Robert Adams
Richard Alberts
Robert Gunning
John H. Head
James S. Thayer
Albert Neal
Alton J. Pierce
T. E. Tawell
George A. Kahler

3. Investigators and Students

Independent Investigators, 1951

Allen, M. Jean, Assistant Professor of Zoology, University of New Hampshire.
Allfrey, Vincent, Assistant, The Rockefeller Institute for Medical Research.
Alscher, Ruth Paula, Assistant Professor of Biology, Manhattanville College of the Sacred Heart.

Amberson, William R., Professor of Physiology, University of Maryland Medical School.
Anderson, Rubert S., Professor of Physiology, University of South Dakota.
Armstrong, Philip B., Professor of Anatomy, State University of New York at Syracuse.
Baily, Norman A., Research Scientist, Columbia University.
Barron, E. S. Guzman, Associate Professor of Biochemistry, University of Chicago.
Bartlett, James H., Professor of Physics, University of Illinois.
Barton, Jay, II, Instructor in Zoology, Columbia University.
Benesch, Reinhold, Fellow in Experimental Medicine, Northwestern University.
Berger, Charles A., Director, Biological Laboratory, Fordham University.
Bevelander, Gerrit, Professor of Histology, New York University.
Bishop, David W., Professor of Physiology, University of Massachusetts.
Bloch, Edward H., Investigator, American Heart Association, Western Reserve University.
Blum, Harold F., Physiologist, N.C.I., and Visiting Lecturer, Princeton University.
Boettiger, Edward P., Assistant Professor of Zoology, University of Connecticut.
Bonner, John Tyler, Assistant Professor of Biology, Princeton University.
Borei, Hans, Visiting Professor, University of Pennsylvania.
Bridgman, Josephine, Agnes Scott College.
Bronk, Detlev W., President, Johns Hopkins University.
Brooks, Matilda M., Research Associate, University of California.
Brown, F. A., Jr., Chairman, Dept. Biological Sciences, Northwestern University.
Brunst, Victor V., Research Associate, University of Maryland Medical School.
Burbanck, W. D., Professor of Biology, Emory University.
Butler, Elmer G., Professor of Zoology, Princeton University.
Charles, Donald R., Chairman, Dept. of Biology, University of Rochester.
Chase, Aurin M., Associate Professor of Biology, Princeton University.
Cheney, Ralph Holt, Professor of Biology, Brooklyn College.
Chitwood, B. G., Associate Professor of Biology, Catholic University.
Ciereszko, Leon S., Assistant Professor of Chemistry, University of Oklahoma.
Claff, C. Lloyd, Research Associate in Surgery, Harvard Medical School.
Clark, Arnold M., Associate Professor of Biology, University of Delaware.
Clark, Eliot R., Professor, *Emeritus,* of Anatomy, University of Pennsylvania.
Clement, A. C., Associate Professor of Biology, Emory University.
Clowes, G. H. A., Research Director, *Emeritus,* Eli Lilly and Company.
Cohen, Adolph I., Graduate Student, Columbia University.
Cohen, Seymour S., Associate Professor Physiol. Chemistry and Pediatrics, Children's Hospital, Philadelphia.
Colwin, Arthur L., Assistant Professor of Biology, Queens College.
Colwin, Laura N., Queens College.
Conklin, Edwin G., Professor of Biology, *Emeritus,* Princeton University.
Cooperstein, Sherwin J., Instructor in Anatomy, Western Reserve University.
Copley, Alfred L., Assistant Clinical Professor of Medicine, New York Medical College.
Cornman, Ivor, Assistant Research Professor in Anatomy, George Washington University School of Medicine.
Costello, Donald P., Kenan Professor of Zoology, University of North Carolina.
Crowell, Sears, Assistant Professor of Zoology, Indiana University.
Curtis, W. C., Professor of Zoology, *Emeritus,* University of Missouri.
Dan, Katsuma, Professor of Biology, Tokyo Metropolitan University, Tokyo, Japan.
Dieter, C. D., Head of Department of Biology, Washington-Jefferson College.
Diller, William F., Assistant Professor of Zoology, University of Pennsylvania.
Doty, Maxwell S., Associate Professor of Botany, University of Hawaii.
Duryee, William R., Cytologist, Carnegie Institution.
Edds, M. V., Jr., Assistant Professor of Biology, Brown University.
Elliott, Alfred M., Professor of Zoology, University of Michigan.
Failla, G., Professor of Radiology, Columbia University.
Fernando, Henry E., Instructor in Entomology, Dept. Agriculture, Ceylon.
Fisher, Joseph D., Research Biochemist, Armour Laboratories, Chicago.
Fries, E. F. B., Assistant Professor, City College of New York.
Gaffron, Hans, Associate Professor of Biochemistry, University of Chicago.

MARINE BIOLOGICAL LABORATORY
WOODS HOLE
LIBRARY
MASS.

Geren, Betty Ben, Research Associate, Massachusetts Institute of Technology.
Gilman, Lauren C., Associate Professor of Zoology, University of Miami.
Goodall, M. C., Research Fellow, Institute for Muscle Research.
Goodchild, Chauncey G., Professor of Biology, S. W. Missouri State College.
Green, James W., Assistant Professor of Physiology, Rutgers University.
Grosch, Daniel S., Associate Professor of Zoology, North Carolina State College.
Grundfest, Harry, Associate Professor of Neurology, College of Physicians and Surgeons.
Guttman, Rita, Assistant Professor, Brooklyn College.
Hajdu, Stephen, Research Fellow, Institute for Muscle Research.
Harvey, Ethel Browne, Independent Investigator, Princeton University.
Harvey, E. Newton, Professor of Physiology, Princeton University.
Haxo, Francis T., Assistant Professor of Biology, Johns Hopkins University.
Heilbrunn, L. V., Professor of Zoology, University of Pennsylvania.
Heller, John Herbert, Chairman, Medical Physics Study Unit, Yale University School of Medicine.
Hendley, Charles D., Associate Professor of Pharmacology, University of South Dakota.
Henley, Catherine, Research Associate, University of North Carolina.
Hodes, Robert, Professor of Neurophysiol. and Exp. Neurology, Tulane University School of Medicine.
Horvath, Beni, Institute for Muscle Research.
Jacob, Miriam I., Graduate Student, New York University.
Jacobs, M. H., Professor of General Physiology, University of Pennsylvania.
Jacobs, William P., Assistant Professor of Biology, Princeton University.
Jenkins, George B., Professor of Anatomy, *Emeritus,* George Washington University.
Jensen, Aage Bohus, Research Fellow, Institute for Cancer Research, Fox Chase, Philadelphia.
Jørgensen, C. Barker, University Adjunct, University of Copenhagen.
Karnovsky, M. L., Associate, Harvard University Medical School.
Karush, Fred, Assistant Professor, Children's Hospital, Philadelphia.
Kelly, Sally, M., Assistant Professor of Plant Science, Vassar College.
Kempton, Rudolf T., Professor of Zoology, Vassar College.
Keosian, John, Professor of Biology, Rutgers University.
Kind, C. Albert, Assistant Professor of Chemistry, University of Connecticut.
King, John W., Professor of Biology, Morgan State College.
Kisch, Bruno, Professor of Chemistry, Yeshiva University.
Kleinholz, Lewis H., Professor of Biology, Reed College.
Klotz, Irving M., Professor of Chemistry and Biology, Northwestern University.
Krahl, Maurice E., Associate Professor of Biochemistry, Washington University School of Medicine.
Kuffler, Stephen W., Associate Professor, John Hopkins University.
Kuntz, Eloise, Instructor in Physiology, Vassar College.
Lansing, Albert I., Associate Professor of Anatomy, Washington University.
Lavin, George I., The Rockefeller Institute for Medical Research.
Layton, Lawrence L., Assistant Professor of Biochemistry, Johns Hopkins University.
Lazarow, Arnold, Associate Professor of Anatomy, Western Reserve University.
LeFevre, Paul G., Associate Professor of Physiology and Biophysics; University of Vermont.
Leveque, Theodore F., Research Assistant, Dept. Anatomy, University of Colorado School of Medicine.
Levy, Milton, Associate Professor of Chemistry, New York University.
Libet, Benjamin, Assistant Professor of Physiology, University of California Medical School.
Lillie, Ralph S., Professor of Physiology, *Emeritus,* University of Chicago.
Lilien, Otto M., Research Fellow, Jefferson Medical College.
Ling, Gilbert N., Instructor in Physiol. Optics, Wilmer Institute, Johns Hopkins Hospital.
Lochhead, John H., Associate Professor of Zoology, University of Vermont.
Loeser, Charles N., Instructor in Anatomy, Western Reserve University.
Love, Warner E., Research Fellow in Biophysics, Johnson Foundation, University of Pennsylvania.
Lovelace, Roberta, Adjunct Professor of Biology, University of South Carolina.

Lucké, Balduin, Professor of Pathology, University of Pennsylvania.
Lynch, William F., Professor of Biology, St. Ambrose College.
Marsland, Douglas, Professor of Biology, New York University, Washington Square College.
Martius, Carl, Professor, Physiol.-chem. Institut, Tübingen, Germany.
Mason, Howard S., Research Associate, Princeton University.
Maxfield, Myles, Research Associate, Massachusetts Institute of Technology.
Mazia, Daniel, Associate Professor of Zoology, University of California.
Metz, Charles B., Assistant Professor of Zoology, Yale University.
Mihalyi, Elemer, Research Fellow, Institute for Muscle Research.
Mitchell, Ruth, Research Associate, Tufts Medical School.
Moore, George M., Professor of Zoology, University of New Hampshire.
Mullins, L. J., Associate Professor, Purdue University.
Musacchia, Xavier J., Instructor, Biological Laboratories, St. Louis University.
Nachmansohn, David, Assistant Professor of Neurology, College of Physicians and Surgeons.
Nelson, Leonard, Instructor of Physiology, University of Nebraska.
Nejelski, Leo L., Jr., Research Associate, Institute for Muscle Research.
Oberholzer, Rudi, Rockefeller Research Fellow, Johns Hopkins University.
Osterhout, W. J. V., Member, *Emeritus*, Rockefeller Institute for Medical Research.
Packard, Charles, Woods Hole, Massachusetts.
Parmenter, Charles L., Professor of Zoology, University of Pennsylvania.
Parpart, Arthur K., Chairman, Department of Biology, Princeton University.
Passano, Leonard M., III, Graduate Student, Yale University.
Peacock, Samuel M., Instructor of Neurophysiology, Tulane University School of Medicine.
Perkins, John F., Assistant Professor of Physiology, University of Chicago.
Pettibone, Marion H., Researcher on Annelids, U. S. National Museum.
Pierce, Madelene E., Professor of Zoology, Vassar College.
Plough, Harold H., Professor of Biology, Amherst College.
Provasoli, Luigi, Chairman of Biology Department, St. Francis College.
Rauch, Harold, Instructor in Zoology, University of Massachusetts.
Rieser, Peter, Instructor in Physiology, Fordham University.
Rice, Mary E., Research Associate, Columbia University.
Ronkin, Raphael R., Assistant Professor of Physiology, University of Delaware.
Rose, S. Meryl, Associate Professor of Zoology, University of Illinois.
Roth, Jay S., Assistant Professor of Biological Chemistry, Hahnemann Medical College.
Rugh, Roberts, Associate Professor in Radiology, Columbia University.
Rutstein, David D., Professor of Preventive Medicine, Harvard Medical School.
Saetren, Hans, Visiting Investigator, The Rockefeller Institute for Medical Research.
Schaeffer, Asa A., Professor of Biology, Temple University.
Schmitt, Francis O., Head of Department of Biology, Massachusetts Institute of Technology.
Schneider, Lillian K., Research Assistant in Microbiology, Columbia University.
Scholander, P. F., Office of Naval Research.
Schulman, Martin P., Research Associate, University of Pennsylvania.
Scott, Allan C., Professor of Biology, Colby College.
Scott, Sister Florence Marie, Professor of Biology, Seton Hill College.
Scott, George T., Associate Professor of Zoology, Oberlin College.
Shanes, Abraham M., Physiologist, National Institutes of Health.
Shaver, John R., Assistant Professor of Zoology, University of Missouri.
Sichel, F., Professor of Physiology and Biophysics, University of Vermont College of Medicine.
Singh, Inderjit, Professor of Physiology, Medical College, Agso, India.
Smith, Ralph I., Assistant Professor of Zoology, University of California.
Speidel, Carl C., Professor of Anatomy, University of Virginia.
Spratt, Nelson T., Associate Professor of Zoology, University of Minnesota.
Steinbach, H. B., Professor of Zoology, University of Minnesota.
Stern, Herbert, Assistant to the Institute, Rockefeller Institute for Medical Research.

Stewart, Dorothy R., Associate Professor and Head of Biology Dept., Rockford College.
Stokey, Alma G., Professor of Plant Science, *Emeritus,* Mount Holyoke College.
Stunkard, Horace W., Professor of Biology, New York University.
Szent-Gyorgyi, Albert, Chief Investigator, Institute for Muscle Research.
Szent-Gyorgyi, Andrew G., Research Fellow, Institute for Muscle Research.
Taylor, Wm. Randolph, Professor of Botany, University of Michigan.
Tobias, Julian M., Associate Professor of Physiology, University of Chicago.
Trager, William, Associate Member, Rockefeller Institute.
Trinkaus, J. P., Assistant Professor of Zoology, Yale University.
Truant, Aldo P., Assistant Professor of Pharmacology, Tufts Medical School.
Villee, Claude A., Assistant Professor of Biological Chemistry, Boston Lying-In Hospital.
Wald, George, Professor of Biology, Harvard University.
Warner, Robert C., Assistant Professor of Chemistry, New York University College of Medicine.
Wichterman, Ralph, Professor of Biology, Temple University.
Wiercinski, Floyd J., Assistant Professor of Physiology, Hahnemann Medical College.
Wilber, Charles G., Director, Biological Laboratories, St. Louis University.
Wilson, Walter L., Instructor in Physiology and Biophysics, University of Vermont, College of Medicine.
Wood, Richard D., Assistant Professor of Botany, Rhode Island State College.
Wright, Paul, Professor of Zoology, University of Michigan.
Wrinch, Dorothy, Lecturer in Physics, Smith College.
Zwilling, Edgar, Associate Professor, University of Connecticut.

Beginning Investigators, 1951

Allen, Margaret D., Graduate Student, University of Pennsylvania.
Allen, Robert Day, Fellow, National Institutes of Health, University of Pennsylvania.
Becker, Hal C., Electronics Engineer (Biophysics), Tulane University School of Medicine.
Berman, Ruth, Graduate Student, Columbia University.
Brummett, Anna R., Graduate Student, Bryn Mawr College.
Chaet, Alfred B., Graduate Student, University of Pennsylvania.
Clark, Marguerite Ruth, Graduate Fellow, St. Louis University.
Davis, Edgar F., Research Assistant, Princeton University.
Fisher, Harriet, Analytical Chemist, Armour Laboratories.
Goldstein, Lester, Assistant Instructor of Zoology, University of Pennsylvania.
Gross, Paul Randolph, Graduate Student and Assistant Instructor in Zoology, University of Pennsylvania.
Herr, Earl B., Jr., Graduate Student, University of Delaware.
Hoffman, Joseph F., Graduate Student, Princeton University.
Honegger, Carol M., Instructor, Temple University.
Inoué, Shinya, Graduate Student, Princeton University.
Jacobson, Alice A., Graduate Student, University of Pennsylvania.
Katz, Michael, Assistant Instructor, University of Pennsylvania.
Mitchell, Constance, Instructor of Biology, University of Delaware.
Moner, John G., Graduate Student, Princeton University.
Peyser, Pincus, Graduate Student, Columbia University.
Proctor, Nathaniel K., Student, University of Pennsylvania.
Ray, David T., Instructor in Zoology, Howard University.
Rudenberg, F. Hermann, Graduate Student, University of Chicago.
Strout, Phyllis M., Student, Goucher College.
Sullivan, Robert Little, Graduate Student, North Carolina State College.
Thomas, Lyell J., Graduate Student, University of Pennsylvania.
Townsley, Sidney J., Graduate Student, Yale University.
Webb, H. Marguerite, Assistant Professor of Biology, Boston College.

Research Assistants, 1951

Adelman, William J., University of Vermont College of Medicine.
Balzer, Dorothy T., Harvard Medical School.
Barber, Saul B., Osborn Zoological Laboratory, Yale University.
Bennett, Michael, Yale University.
Bernatowicz, Albert J., University of Michigan.
Brumm, Anne F., Harvard Medical School.
Bullowa, Anne, New York Medical College.
Burchard, John E., Princeton University.
Chapman, George B., National Cancer Institute.
Clendenning, K. A., National Research Council of Canada.
Cohen, Max, Columbia University.
Cowdry, Alice M., Wellesley College.
Cross, Helen, Columbia University.
Currier, Joanne, Vassar College.
Curtiss, Ruth A., Indiana University.
Donovan, Joanne E., Yale University.
Druva, Emily, Columbia University.
Fingerman, Milton, Northwestern University.
Gagnon, André, University of Pennsylvania.
Gillespie, Ruth J., Carnegie Institution.
Greengard, Paul, Johns Hopkins University.
Guyselman, J. Bruce, Northwestern University.
Hines, Margaret N., Northwestern University.
Hodge, Alan J., Massachusetts Institute of Technology.
Holland, Janice K., Brown University.
Howard, Robert S., Northwestern University.
Iampietro, P. F., University of Massachusetts.
Jacobson, Marcus A., White Plains, New York.
Johnson, Phyllis E., University of Chicago.
Kauzmann, Mrs. Elizabeth, National Cancer Institute.
Kaye, Alvin M., Columbia University.
Kent, Donald E., University of North Carolina.
Landau, Joseph V., New York University.
Leonard, Lawrence M., Harvard University.
Ling, Chiun-Tong, Johns Hopkins University.
Martin, B. Jean, University of Minnesota.
McIntyre, Jane H., Harvard Medical School.
Merz, Timothy, Johns Hopkins University.
Milstein, Seymour W., Hahnemann Medical College.
Murray, Albert E., University of Illinois.
Nemeth, Andrew, Johns Hopkins University School of Medicine.
Padawer, Jacques, New York University.
Pauling, Peter J., Columbia University.
Robinson, Elizabeth B., Amherst College.
Ruszel, S. Frances, Rockefeller Institute for Medical Research.
Sandeen, Muriel I., Duke University.
Seki, Sada Louise, University of Chicago.
Sights, Warren P., University of Chicago.
Silberger, Julius, Jr., Harvard College.
Slater, John V., University of Michigan.
Tietze, Frank, Washington University.
Whitcomb, John, Amherst College.

Library Readers, 1951

Ambrus, Julian L., Associate Professor, Philadelphia College of Pharmacy and Science.
Botsford, E. Frances, Professor of Zoology, Connecticut College.
Brackett, Sterling, Manager of Technical Service, Lederle Laboratories.
Burwell, E. Langdon, Practicing Physician, Woods Hole.
Davis, Bernard D., Surgeon, Tubercular Research Lab., Cornell University Medical College.
DeKornfeld, Thomas J., Chevy Chase, Maryland.
DeLamater, Edward D., Research Professor, University of Pennsylvania.
Dorfman, Albert, Assistant Professor of Pediatrics, University of Chicago Clinics.
DuBois, Eugene F., Professor of Physiology, *Emeritus*, Cornell University Medical College.
Eichel, Bertram, Assistant Research Specialist, Rutgers University.
Eichel, Herbert J., Research Fellow, Rutgers University.
Gabriel, Mordecai L., Assistant Professor of Biology, Brooklyn College.
Gurevich, Vladimir, Assistant Visiting Physician, Bellevue Hospital.
Kabat, Elvin A., Associate Professor of Bacteriology, College of Physicians and Surgeons.
Kauzman, Walter, Assistant Professor of Chemistry, Princeton University.
Keeffe, Mary M., Assistant Professor of Biology, College of St. Thomas.
Kramer, Mollie P., Bibliographer, American Meteorological Society.
Lachance, Jean Paul, Research Assistant, Laval University.
Loewdin, Per-Olov, Research Associate, Duke University.
Loewi, Otto, Research Professor of Pharmacology, New York University.
McDonald, Sister Elizabeth Seton, College of Mount St. Joseph-on-the-Ohio.
Marinelli, Leonidas D., Senior Biophysicist, Argonne National Laboratory.
Memhard, Allen R., Riverside, Connecticut.
Meyerhof, Otto, Research Professor, University of Pennsylvania School of Medicine.
Neuberg, Carl, Research Professor, Polytechnic Institute.
Person, Philip, Research Fellow, Rutgers University.
Pettit, Lincoln C., Head of Biology Department, Hiram College.
Root, Walter S., Professor of Physiology, College of Physicians and Surgeons.
Roys, Chester, Research Associate, Tufts College.
Schlesinger, R. Walter, Associate Member, Public Health Research Inst. of the City of New York.
Schmidt-Nielsen, Knut, Assistant Professor, University of Cincinnati.
Shwartzman, Gregory, Head of Dept. of Bacteriology, Mount Sinai Hospital.
Stern, Kurt G., Adjunct Professor of Biochemistry, Polytechnic Institute.
Sulkin, S. Edward, Professor and Chairman, Dept. Bacteriology, Southwestern Medical School.
Tamm, Olga, Assistant Librarian, The American Geographical Society of New York.
Tamm, Igor, Assistant, Rockefeller Institute for Medical Research.
Wainio, Walter W., Associate Professor of Biochemistry, Rutgers University.

Students, 1951

BOTANY

Donahue, Barbara Anne, Smith College.
Groesbeck, Marjorie E., Elmira College.
Hooper, Mary Elizabeth, Elmira College.
Jennings, Peter Randolph, Drew University.
Joseph, T. C., Fordham University.
Masselink, Sally Ann, Vassar College.
Newhouse, W. Jan, University of New Hampshire.
O'Brien, Muriel J., Washington, D. C.
Pinkney, Addison Vastapha, Jr., Morgan State College.
Sinski, James Thomas, Marquette University.
Szerlip, Martha, Smith College.
Trefz, Shirley M., Temple University.
Wilce, Robert Thayer, University of Vermont.

EMBRYOLOGY

Anderton, Laura Gaddes, Woman's College of the University of North Carolina.
Angevine, Jay Bernard, Cornell University.
Behrman, Edward Joseph, Yale University.
Berger, Samuel Harold, Columbia University.
Byers, Helen Louise, Indiana University.
Castleman, Mary Elizabeth, Randolph-Macon Woman's College.
Collier, Jack Reed, University of North Carolina.
Dossel, William Edward, Johns Hopkins University.
Fauteux, Muriel Doris, University of Massachusetts.
Forsthoefel, Paulinus F., Ohio State University.
Gall, Joseph G., Yale University.
Greene, Peter H., Amherst College.
Jacobson, Antone G., Harvard College.
Jenkins, Floyd Albert, St. Louis University.
Leib, Gilbert M. P., Haverford College.
Livingstone, Katherine Elizabeth, Acadia University.
McGregor, Ina Louise, Clark University.
Piavis, George Walter, Westminster, Maryland.
Potter, David Dickinson, Swarthmore College.
Steinberg, Malcolm Saul, Amherst College.
Subtelny, Stephen, University of Missouri.
Tamar, Henry, New York University.
Watt, Donald James, Wesleyan University.
Weisheit, Phyllis Jeanette, University of Wisconsin.
Whallon, Jeanne, Indiana University.
Wood, Dorothy Evelyn, Marquette University.
Youngs, Lillian Margot, University of North Carolina.

PHYSIOLOGY

Ames, Bruce Nathan, California Institute of Technology.
Baron, Jean Bernard, Columbia University.
Battley, Edwin H., Stanford University.
Budenstein, Zelda, Mount Holyoke College.
Cori, Osvaldo, University of Chile.
Davis, LeRoy Thomas, Syracuse University.
Egan, Richard, University of Maryland School of Medicine.
Eichler, Myron Franklin, State University of New York at New York.
Ericson, Hartley Charles, Northwestern University.
Feldman, Donald, Tulane University.
Garlington, Laurens Nelson, Duke University.
Gollub, Seymour, Mt. Sinai Hospital.
Groth, Donald P., University of Wisconsin.
Hall, Kenneth Deland, Duke University Medical School.
Keane, John Francis, Jr., St. Louis University.
Killam, Iona Claire, Acadia University.
King, Donald W., Columbia University.
Landau, Joseph Victor, New York University, Washington Square College.
Levine, Celia, Columbia University.
Mayo, Mercedes Diaz, Universidad de la Habana.
Melton, Carlton Earl, Jr., University of Illinois.
Overman, Ted, Louisiana State University School of Medicine.
Pyle, Zoula Pauline, State University of Iowa.
Resnik, Robert Alan, Purdue University.
Robyns, Rose, Rochester Medical School.
Smithberg, Morris, University of Rochester.

Thurlow, Jane Odiorne, Rutgers University.
Walsh, Raymond R., Cornell University.
Welch, Claude Alton, Michigan State College.

INVERTEBRATE ZOOLOGY

Anderson, Paul Knight, Orange, Massachusetts.
Bacha, William J., Jr., Long Island University.
Banks, Edwin M., University of Chicago.
Bennett, Michael, Yale University.
Berberian, Joseph Francis, University of Notre Dame.
Bernstein, Paul William, Washington University.
Buch, Florence Grace, Washington Square College.
Clark, Martha Grace, DePauw University.
Cowperthwaite, Jean, New York University.
Dearden, Lyle C., University of Massachusetts.
Fesco, Edward J., Villanova College.
Fleming, T. Corwin, Harvard College.
Fleminger, Abraham, Harvard University.
Flinn, Louise Croswell, Huntingdon College.
Flori, Beatrice Marian, University of Illinois.
Frederickson, Jean, Oberlin College.
Greenstein, Julius Sidney, University of Illinois.
Hill, Robert Benjamin, Tufts College.
Hills, Alicia M., Radcliffe College.
Hull, Robert W., University of Illinois.
Kennedy, Donald, Harvard University.
King, Elizabeth Norfleet, Wellesley College.
Kirner, Stephen H., Hamilton College.
Kurland, Aaron, University of Illinois.
Lacey, Richard J., University of Illinois.
LaDage, Barbara Joan, Northwestern University.
LaFleur, Rev. Angelus, Fordham University.
Leeds, Mary M., Antioch College.
Legault, Rev. Romeo O., University of Ottawa.
Loudermill, Phyllis Ann, Hood College.
Macomber, Kent Jean Oakley, Vassar College.
Margez, Alphonsus, Catholic University.
Marzullo, Carmela Maria, Wesleyan University.
Mathies, Allen Wray, Jr., Colorado College.
McGill, David A., Bucknell University.
Parsons, Edward Lewis, University of Massachuetts.
Paterson, Mabel, University of Illinois.
Paulsen, Elizabeth, Rutgers University.
Payne, Charles B., Jr., Yale University.
Plaine, Henry LeRoy, Johns Hopkins University.
Platz, Susan Cartwright, University of Illinois.
Pratt, Anne E., Wilson College.
Rollman, Irene, Hunter College.
Rondon, Teresa, Smith College.
Sacks, Martin, University of Illinois.
Sinden, Mabel, University of Minnesota.
Sohn, I. G., U. S. Geological Survey.
Spielman, Andrew, Colorado College.
Stemler, Fred Walter, Purdue University.
Teal, John Moline, Harvard University.
Thimann, Vivianne, Swarthmore College.
Warren, Patricia, West Newton, Massachusetts.

WOOD, EUNICE M., Wellesley College.
WORTMAN, DIANA, Brooklyn College.
YARMOLINSKY, MICHAEL BEZALEL, Johns Hopkins University.

4. THE LALOR FELLOWS, 1951

BARTON, JAY, II, Columbia University.
BENESCH, REINHOLD, Northwestern University.
CIERESZKO, LEON S., University of Oklahoma.
COHEN, SEYMOUR S., Children's Hospital of Philadelphia.
DAN, KATSUMA, Tokyo Metropolitan University.
JACOBS, WILLIAM P., Princeton University.
JØRGENSEN, C. BARKER, University of Copenhagen.
KARNOVSKY, M. L., Harvard University Medical School.
KELLY, SALLY M., Vassar College.
SCHULMAN, MARTIN P., University of Pennsylvania.

5. TABULAR VIEW OF ATTENDANCE, 1947–1951

	1947	*1948*	*1949*	*1950*	*1951*
INVESTIGATORS—Total	299	326	344	338	303
Independent	182	183	193	198	186
Under Instruction	36	42	52	43	28
Library Readers	36	50	55	48	37
Research Assistants	45			49	52
STUDENTS—Total	131	123	128	126	124
Zoology	55	54	55	55	55
Embryology	33	29	31	29	27
Physiology	26	25	27	27	29
Botany	17	15	15	15	13
TOTAL ATTENDANCE	430	449	472	444	427
Less persons registered as both students and investigators	2	6	2		
	428	443	470	444	427
INSTITUTIONS REPRESENTED—Total	148	158	155	156	158
By Investigators	114	117	114	114	115
By Students	56	68	68	67	43
SCHOOLS AND ACADEMIES REPRESENTED					
By Investigators	1	1	1	2	1
By Students	1				1
FOREIGN INSTITUTIONS REPRESENTED					
By Investigators	7	8	6	6	8
By Students	3	4	3	2	3

6. COOPERATING AND SUBSCRIBING INSTITUTIONS, 1951

Cooperating Institutions

Amherst College
Brooklyn College
Bryn Mawr College
Children's Hospital of Philadelphia
College of Mt. St. Joseph-on-the-Ohio
College of Physicians and Surgeons
Columbia University
Drew University
Duke University
Elmira College
Fordham University
Goucher College
Harvard University
Harvard University Medical School
Hunter College
Institute for Muscle Research

Johns Hopkins University
Johns Hopkins University Medical School
Eli Lily and Company
Massachusetts Institute of Technology
Morgan State College
Mount Sinai Hospital
National Cancer Institute
New York University College of Medicine
New York University, Heights
New York University, Washington Square College
North Carolina State College of Agriculture and Engineering
Northwestern University
Oberlin College
Princeton University
Rockefeller Institute for Medical Research
Rutgers University
Saint Louis University
Smith College
Southwestern Medical College
State College of New York, School of Medicine
Temple University
Tufts College Medical School
Union College
University of Chicago
University of Connecticut
University of Delaware
University of Illinois
University of Kansas
University of Maryland School of Medicine
University of Massachusetts
University of Michigan
University of Minnesota
University of Missouri
University of New Hampshire
University of Oklahoma
University of Pennsylvania
University of Pennsylvania Medical School
University of Vermont Medical School
University of Virginia
University of Wisconsin
Vassar College
Washington University
Wesleyan University
Western Reserve University
Yale University

Subscribing Institutions

Acadia University
Catholic University
Hahnemann Medical School
Indiana University
Jefferson Medical College
Long Island University
Marquette University
National Research Council
Public Health Research Institute of New York City
Radcliffe College
Tulane University School of Medicine
University of North Carolina
University of Rochester
University of South Dakota
Wellesley College

7. Evening Lectures

The Friday Evening Lectures, 1951

Friday, June 29
Dr. Douglas A. Marsland .... "Gelation in Relation to Protoplasmic Contractility."

Friday, July 6
Dr. Severo Ochoa .... "Carbon Dioxide Fixation in Animals and Plants."

Friday, July 13
Dr. Ralph W. Gerard .... "Neural Basis of Behaviour."

Friday, July 20
Dr. Balduin Lucké .... "Tumors in Cold-blooded Vertebrates. Their Significance in the Experimental Study of Cancer."

Friday, July 27
Dr. Hans Borei .... "Respiration of the Egg Cell Before and After Fertilization."

Friday, August 3	
Dr. Denis L. Fox	"Colloidal Materials in the Nutrient Cycle of the Sea."
Friday, August 10	
Dr. Nelson T. Spratt, Jr.	"Nutritional and Environmental Requirements for Development of the Early Chick Embryo."
Friday, August 17	
Dr. T. H. Waterman	"The Physiology of the Compound Eye."
Friday, August 24	
Dr. R. W. G. Wyckoff	"Macromolecular Particles and Viruses in Tissues of Plants and Animals."

Other Lectures

Monday, August 13	
Dr. Katsuma Dan	"Cyto-embryology in the Sea Urchin."

8. Seminars, 1951

July 3	
B. Libet, H. J. Ralston and B. Feinstein	"Effect of Stretch on Action Potentials of Muscle."
L. S. Ciereszko	"An Application of Ion-Exchange Resins to the Isolation of Amino Acids from Biological Materials."
J. T. Bonner	"Morphogenesis of the Slime Bacterium, *Chondromgees.*"
July 10	
H. S. Mason and E. Davis	"Status of Luciferin Chemistry."
Claude A. Villee and Dorothy Balzer	"Studies of the Metabolism of Fetal and Placental Tissues."
Wm. P. Jacobs	"The Nature of the Hormonal Factor Which Normally Limits the Differentiation of a Specific Cell-Type."
July 17	
Reinhold Benesch and Ruth E. Benesch	"Studies on Biologically Occurring Mercaptans and Some of Their Mercaptides."
Charles G. Wilber	"Blood and Liver Lipids in the Northern Pike."
Gerrit Bevelander	"Calcification in Molluscs."
July 24	
Sidney Solomon and Julian Tobias	"Long Axis Movement of Nerve Ca, K, and Na in an Electric Field."
Rudi Oberholzer	"Influence of Various Potassium Concentrations on Resting Respiration of Excised Frog Nerve."
I. B. Wilson	"Reaction of Substrates and Inhibitors With the Surface of Acetylcholinesterase."

D. Nachmansohn	"The Curare Effect and Axonal Conduction."
July 31	
Ivor Cornman	"Specificity of Steroids Which Inhibit Heart Beat."
Edward G. Boettiger and E. Furshpan	"Observations of the Flight Mechanism of Flies."
David R. Stadler	"Chemotropism in *Rhizopus nigricans:* The Staling Reaction."

9. Members of the Corporation, 1951

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Calvert, Dr. Philip P., University of Pennsylvania, Philadelphia, Pennsylvania.
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Conklin, Prof. Edwin G., Princeton University, Princeton, New Jersey.
Cowdry, Dr. E. V., Washington University, St. Louis, Missouri.
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King, Mr. Chas. A.
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Moore, Dr. J. Percy, University of Pennsylvania, Philadelphia, Pa.
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Strong, Dr. O. S., Columbia University, New York City, New York.
Waite, Prof. F. C., 144 Locust Street, Dover, New Hampshire.
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Adolph, Dr. Edward F., University of Rochester Medical School, Rochester, New York.
Albaum, Dr. Harry G., Biology Dept., Brooklyn College, Brooklyn, N. Y.
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Anderson, Dr. T. F., University of Pennsylvania, Philadelphia, Pennsylvania.

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Ball, Dr. Eric G., Department of Biological Chemistry, Harvard University Medical School, Boston, Massachusetts.

Ballard, Dr. William W., Dartmouth College, Hanover, New Hampshire.

Bard, Prof. Philip, Johns Hopkins Medical School, Baltimore, Maryland.

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Bartlett, Dr. James H., Department of Physics, University of Illinois, Urbana, Illinois.

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Bigelow, Prof. R. P., Massachusetts Institute of Technology, Cambridge, Massachusetts.

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Blum, Dr. Harold F., Department of Biology, Princeton University, Princeton, New Jersey.

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THE EFFECTS OF DIFFERENT HALOGENATED ALKYL AMINES ON THE DIVISION OF SEA URCHIN EGGS[1]

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With a view to obtaining some insight into the cytological effects of a representative series of nitrogen mustards, 27 compounds, mostly β-chloroethyl amines, were tested for their ability to block or retard the cleavage of sea urchin eggs.

With such eggs, each exposed directly to the experimental solution, there is unexcelled opportunity to study the response of the cell to the mustard without concern for effects of a multicellular organism on the mustard, and on the response of the cell. With regard to mitosis, the uniform, controllable division of the eggs makes possible the application of the agent to any phase of cleavage, and ready analysis of alterations in each phase.

MATERIALS AND METHODS

For the over-all survey, the eggs of *Arbacia punctulata* were used. Some supplementary studies employed the eggs of *Tripneustes esculentus,* and for detailed observations of the course of events during inhibition of mitosis, the transparent eggs of *Lytechinus variegatus* were used.

For routine comparison of activities, exposure was begun 10–13 minutes after fertilization. Retardation was measured by making a count of 50 eggs 4 to 10 times during the first cleavage and again during the second. The points were connected by straight lines to determine the approximate time of 50 per cent cleavage (*cf.* Fig. 1), and comparison was made between the treated and the control from the same lot of eggs.

Curves for duplicate controls usually gave identical 50 per cent cleavage times, and they seldom differed by more than two minutes, so statistical analysis was not needed. The eggs were followed until they formed plutei, and a record was kept of the time at which they became blastulae and gastrulae. The ranges of response were condensed into groups described in connection with Table II.

The compounds (Table I) were dissolved in sea water and were added to the eggs within two minutes of dissolving. They were supplied to the Sloan-Kettering Institute by Parke-Davis and Company (A); Toxicity Laboratory, The University of Chicago (B); Dr. Stein, Rockefeller Institute (C); Merck and Company (D); Eli Lilly and Company (E); and American Cyanamid Company (F).

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Results and Discussion

Arbacia

The nature of the response of dividing eggs to nitrogen mustard at moderately effective doses is shown in Figure 1. Lower concentrations caused less retardation or affected only the second division or even later stages, while higher concentrations prevented division in all or part of the population.

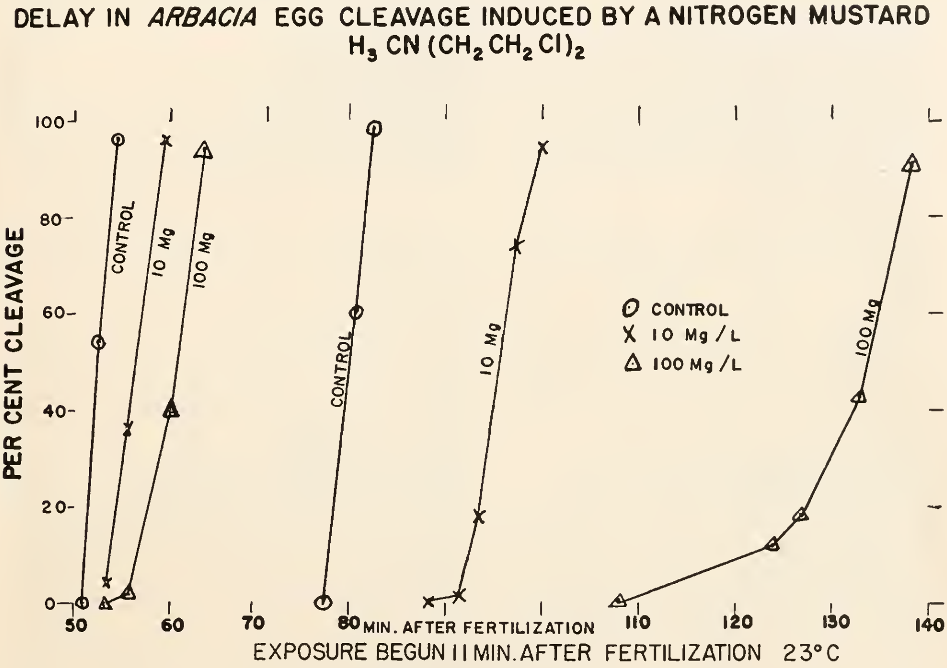
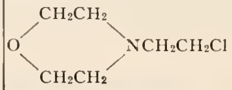
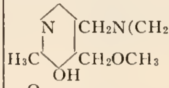
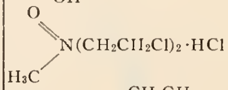
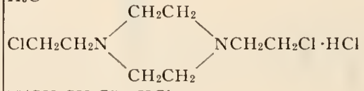
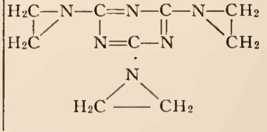
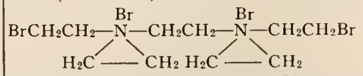


FIGURE 1. The three curves on the left represent first cleavage, the others second cleavage.

The different degrees of retardation and blocking of Arbacia-egg cleavage are shown in Table II, beginning with the most active mustard at the top. Concentrations are expressed as millimols per liter. The grades of effect are the same as those used to quantitate effects of carbamates (Cornman, 1950). Where development was not completely blocked, retardation of the first cleavage was determined from graphs like Figure 1 and then placed in one of three grades: 100% or more, 10% to 99%, or less than 10% increase in the time required to reach 50% first cleavage as compared with the controls. Weaker effects were detected as retardation of second cleavage or of embryogenesis. At 23° ± 0.5° C., the temperature used for all Arbacia experiments, 50 per cent first cleavage was reached usually at 52–53 minutes, but in one experiment was as early as 50 minutes and in another as late as 61 minutes. Second cleavage reached 50 per cent at 84–85 minutes, with extremes at 80 and 93 minutes.

TABLE I

Names and structures of compounds studied

No.	Name	Structure	Source
	β-chloroethylamines		
1	*β*-chloroethyl amine. HCl	$NH_2CH_2CH_2Cl\cdot HCl$	A
2	N-(*β*-chloroethyl)-morpholine		B
3	Dibenzyl-*β*-chloroethyl amine. HCl	$(\varnothing CH_2)_2NCH_2CH_2Cl\cdot HCl$	B
4	*Bis*-(*β*-chloroethyl)-amine. HCl	$NH(CH_2CH_2Cl)_2\cdot HCl$	A
5	Methyl-*bis*-(*β*-chloroethyl)-amine. HCl	$H_3CN(CH_2CH_2Cl)_2\cdot HCl$	B
6	Propyl-*bis*-(*β*-chloroethyl)-amine	$CH_3CH_2CH_2N(CH_2CH_2Cl)_2$	B
7	*n*-amyl-*bis*-(*β*-chloroethyl)-amine. HCl	$CH_3(CH_2)_4N(CH_2CH_2Cl)_2\cdot HCl$	D
8	*n*-hexyl-*bis*-(*β*-chloroethyl)-amine. HCl	$CH_3(CH_2)_5N(CH_2CH_2Cl)_2\cdot HCl$	D
9	*γ*-phenylpropyl-*bis*-(*β*-chloroethyl)-amine. HCl	$\varnothing CH_2CH_2CH_2N(CH_2CH_2Cl)_2\cdot HCl$	D
10	*p*-octylphenoxyethoxyethyl-*bis*-(*β*-chloroethyl)-amine. HCl	$C_8H_{17}\varnothing OCH_2CH_2OCH_2CH_2N(CH_2CH_2Cl)_2\cdot HCl$	D
11	3-*bis*-(*β*-chloroethyl)-aminomethyl-4-methoxy-methyl-5-hydroxy-6-methyl-pyridine. 2HCl	 2HCl	D
12	Methyl-*bis*-(*β*-chloroethyl)-amine oxide. HCl		D
13	N,N′-*bis*-(*β*-chloroethyl)-1,4-piperazine. HCl		D
14	*Tris*-(*β*-chloroethyl)-amine. HCl	$N(CH_2CH_2Cl)_3\cdot HCl$	B
15	N,N,N′,N′-*tetrakis*-(*β*-chloroethyl)-ethylene-diamine. 2HCl	$(ClCH_2CH_2)_2NCH_2CH_2N(CH_2CH_2Cl)_2\cdot 2HCl$	B
16	N,N,N′,N′-*tetrakis*-(*β*-chloroethyl)-propane-diamine. 2HCl	$(ClCH_2CH_2)_2NCH_2CH_2CH_2N(CH_2CH_2Cl)_2\cdot 2HCl$	B
17	N,N,N′,N′-*tetrakis*-(*β*-chloroethyl)-*β*-chloropropane-diamine. 2HCl	$(ClCH_2CH_2)_2NCH_2CHClCH_2N(CH_2CH_2Cl)_2\cdot 2HCl$	B
18	Composition uncertain. Probably a polymer of *bis*-(*β*-chloroethyl)-amine with M.W. 764	$H_2[HCN(CH_2CH_2Cl)_2]_n\cdot nHCl$	D
	Other Chloroalkylamines		
19	Ethyl-*γ*-chloropropyl-*β*-chloroethylamine. HCl	$ClCH_2CH_2N(CH_2CH_3)CH_2CH_2CH_2Cl\cdot HCl$	E
20	*Tris*-(*β*-chloro-*n*-propyl)-amine. HCl. ½H₂O	$N(CH_2CHClCH_3)_3\cdot HCl\cdot \frac{1}{2}H_2O$	D
21	Triethylenimino-*s*-triazine		F
	Other Haloalkylamines		
22	Methyl-*bis*-(*β*-bromoethyl)-amine. HBr	$CH_3N(CH_2CH_2Br)_2\cdot HBr$	D
23	N,N′-(*β*-bromoethyl)-N,N′-(*β*-ethylene-imonium-bromide)-ethylene-diamine		D
24	*n*-butyl-*β*-fluoroethylamine. HCl	$CH_3CH_2CH_2CH_2NHCH_2CH_2F\cdot HCl$	D
25	*n*-butyl-*bis*-(*β*-fluoroethyl)-amine. HCl	$CH_3CH_2CH_2CH_2N(CH_2CH_2F)_2\cdot HCl$	D
	Substituted Ammonium Chlorides		
26	*bis*-(*β*-chloroethyl)-dimethyl-ammonium chloride	$(CH_3)_2NCl(CH_2CH_2Cl)_2$	B
27	tetra-(*β*-chloroethyl)-ammonium chloride	$(ClCH_2CH_2)_2NCl(CH_2CH_2Cl)_2$	C

This series of mustards divides roughly into two groups, the majority blocking cleavage at a few millimols per liter, and retarding the first division at the tenths or hundredths millimolar level (down to No. 13, Table II). About half of this group, including the more typical nitrogen mustards (8, 15, 7, 9, 14, 6, 22), are somewhat more active than the others, increasing cleavage time 100 per cent or more at 0.3–0.5 mM/L and 10–99 per cent at 0.03–0.05 mM/L. The quick effectiveness of *n*-hexyl-*bis*-(β-chloroethyl)-amine (No. 8), preventing all division at 0.38 mM/L, suggests that the six-carbon chain is about optimal for penetration, as against the longer and shorter groups tested.

TABLE II

Relative potencies (mM/L) of halogenated alkyl amines in preventing or retarding Arbacia egg cleavage

Compound No.	Millimolarity required to induce:						
	Complete block (Aa)	Retardation					No effect
		First cleavage			Second cleavage (Cd)	Embryo (D)	
		100% (Ca)	10–99% (Cb)	<10% (Cc)			
8	0.38		.038	.019 .008			
15	2.61	.261	.026				
7	4.02	.402	.040				
9	3.37	.337	.034				
14	4.15	.415	.042				
6	5.44	.544	.544	.054			
22	3.07		.307 .031				
20	3.43		.343	.034			
12			.481	.048			.005
5	5.21		.521	.052			
19			4.52 .452	.045			
10	2.20			.220	.022		
18		1.31		.131	.013		
13	4.05				.405		
3				.338			.034
2			6.85	.685			
1			8.62	.862 .086			
27			3.29				.329
4			11.17	5.59 .559 .056			
26						4.83 .483	
23			20.49				2.05

Aa: No nuclear or cytoplasmic division. C: The time between fertilization and 50% first cleavage increased by 100% (Ca), by 10–99% (Cb), or by less than 10% (Cc). Cd: First cleavage not retarded but second cleavage retarded. D: First two cleavages not retarded but the blastula, gastrula, or pluteus retarded.

Two potent mustards deviate from the typical structural pattern. Methyl-*bis*-(β-chloroethyl)-amine oxide (No. 12) has its nitrogen occupied by oxygen in addition to the alkyl chains. Attachment of the β-chloroethyl radicals to separate nitrogens (No. 13) also forms an active molecule, as pointed out by Goldacre, Loveless and Ross (1949). A terminal chlorine (No. 19) or a β-chlorine (No. 20) in a propyl chain appears to function as well as an ethyl β-chlorine, although Haddow, Kon and Ross (1948) find the γ chlorine inactive in chloroalkylaryl amines. The poor performance of No. 4 is under suspicion, particularly since these compounds hydrolyze readily in the moist seaside atmosphere. A fresh sample was not available for re-checking.

Mustards with only a single β-chloroethyl group (Nos. 1, 2, 3) are relatively inactive, as reported by Haddow, Kon and Ross (1948). On the other hand, addition of a third β-chloroethyl group (14) or even a second substituted nitrogen (15) does not confer outstanding potency on the molecule.

Tripneustes

Experiments with another species at the same temperature show some differences in response, and, because the egg is unpigmented, yield somewhat more in the way of cytological detail.

For comparison, the column headings used for Arbacia (Table II: Ca, Cb, etc., indicating the extent of retardation) are similarly used for Tripneustes in Table III. An additional category, *Incomplete block,* encompasses more severe effects where less than 5 per cent of the eggs divided (b) or where they did not divide at all, but unlike completely blocked eggs, they formed temporary furrows or underwent nuclear division in the absence of cytoplasmic division (Ba). Comparison is made at the time of 50 per cent cleavage which was at 86–101 minutes for the first, and 131–182 minutes for the second division at 23°, or 65–82 minutes for the first and 101–122 minutes for the second at 25°–26° C.

Some distinctive cytological responses were observed in the living eggs. In 0.521 mM of methyl-*bis*-(β-chloroethyl)-amine (No. 5), asters and spindle remained distinct during the 1½ hours that cleavage was delayed and persisted even longer in eggs which did not divide, eventually producing tetranucleate eggs at 6 hours. At 0.052 mM, 60% of the eggs furrowed, but the furrows retracted in half of these. Persistent, often multiple, mitotic figures were also seen in compounds 15 and 24.

In comparing the effects of Nos. 5, 14 and 15 with the less severe damage to Arbacia eggs, it should be kept in mind that the length of exposure before the first cleavage is nearly twice as long in Tripneustes, which divides at about 1½ hours as against 50–60 minutes for Arbacia at 23° C.

No. 24 has only a single fluoroethyl radical, and, as we have seen from Arbacia studies, such "hemi-mustards" are relatively ineffective. However, No. 24, although having both β-fluoroethyl groups, is considerably less toxic than the chloroethylamines. The difference cannot as yet be assigned entirely to the halogen inasmuch as the first five (14, 15, 16, 17, 5) were tested at Bermuda at 23° C. and the last three (11, 25, 24) at Bimini at 25°–26° C. There are also differences among different experiments with the same compound, as shown by diverse severity of effects from identical concentrations of Nos. 15 and 24. These differences arise from differences in the numbers of eggs in separate experiments and probably from differences in the individual urchins.

Lytechinus

In this species 50 per cent cleavage is reached at 46–61 minutes and the second at 70–83 minutes at 25°–27° C.

Triethylenimino-s-triazine

Triethylene melamine is a name commonly applied to compound No. 21. Although lacking the β-halogen, it already carries the imine rings which are formed by some chloroethylamines in solution (Golumbic *et al.*, 1946) and it resembles nitrogen mustards at least in its ability to retard leukemia (Burchenal, *et al.*, 1950) and solid tumors (Lewis and Crossley, 1950), and its effects on cell division (Rose, Hendry and Walpole, 1950).

Table III

Relative potencies (mM/L) of halogenated alkyl amines in preventing or retarding Tripneustes egg cleavage

Compound No.	Millimolarity required to induce:								
	Block			Retardation					No effect
	Complete (Aa)	Incomplete		First cleavage			Second cleavage (Cd)	Embryo (D)	
		(Ba)	(Bb)	100% (Ca)	10–99% (Cb)	<10% (Cc)			
14	0.415			0.042		0.004			0.0004
15		0.261		0.261	0.026	0.026 0.003			0.003 0.0003
16			0.252		0.025 0.003				0.0002
17					0.232 0.023		0.002		0.0002
5				0.521	0.052	0.005			0.0005
11	2.630				0.500 0.263 0.250		0.025		
25					4.975	0.498		0.050	
24					6.430	0.643	6.430	0.064	

Aa: No cytoplasmic or nuclear division. Ba: Evanescent furrows or nuclear division in the absence of cytoplasmic division. Bb: Less than 5% of the eggs divided. C: The time between fertilization and 50% first cleavage increased by 100% (Ca), by 10–99% (Cb), or by less than 10% (Cc). Cd: First cleavage not retarded but second cleavage retarded. D: First two cleavages not retarded but the blastula, gastrula, or pluteus retarded.

At 9.8 mM/L it retarded the first cleavage 35–42 minutes (Cb) and prevented completion of the second cleavage. Half this dose retarded the first cleavage 25–30 minutes (Cb) and the second cleavage 1–2 hours. The second division was remarkably unequal, producing some blastomeres ¼ and ⅓ the diameter of their sisters. At 0.49 mM/L the first cleavage was retarded 4 minutes and the second 29 minutes, but the embryos died before becoming blastulae. Retardation of the first cleavage was one minute and the second 9 minutes at 0.049 mM/L. These became abnormal gastrulae.

Triethylenimino-*s*-triazine thus resembles the mustards in producing extreme retardation at comparable doses, but is unlike them in that concentrations which produce slight retardations are eventually lethal.

Dose-effect relationships with HN2

For the more detailed study of dose-effect relations and cytological effects, methyl-*bis* (β-chloroethyl)-amine (Compound 5, often called HN2) was chosen as a typical mustard. The Lytechinus egg was used because the nucleus and mitotic figure can be clearly observed in the living egg.

TABLE IV

Relationships between concentration of HN2 and inhibition of cleavage in Lytechinus eggs

	mM/L	% First cleavage		Retardation (minutes)		% Increase in cleavage time	
		Count	% Inhib.	1st cl.	2nd cl.	1st cl.	2nd cl.
Expt. III	8.320	2	98				
	4.160	22	76				
	2.080	46	49	40	210	63	248
	1.040	68	24	32	70	50	83
Expt. I	2.080	36	60	38	180	68	254
	1.040	90	0	9½	54½	17	66
	0.521			4	37	7	45
	0.260			0	21		25
	0.130			0	15½		19
	0.065			0	7		8
	0.032			0	1½		2

Expt. III: Control first cleavage 63½ min.; second cleavage 84½ min.
Expt. I: Control first cleavage 55½ min.; second cleavage 82½ min.
Percentage inhibition based on 90% control cleavage in both experiments.

The percentages of Table IV show that the curve for blocking (2–8 mM) is steeper than for retardation (0.03–1.0 mM), suggesting that two mechanisms are involved. Quite possibly cytoplasmic division is affected separately at the higher concentrations, a separation which was visible in eggs where cleavage was suppressed but where the achromatic figure was still active and the nucleus sometimes divided. Higher doses were required to produce effects comparable to those obtained with Arbacia and Tripneustes. These experiments were run at Bimini at temperatures of 24.7°–25.8° C., so environmental as well as species differences may enter in.

"Prophase block"

Some investigators find that the prophase is particularly susceptible to mustard and other "radiomimetic" poisons. In sea urchin eggs, no one phase appeared to be selectively hit. Rather, the entire mitotic sequence was slowed. In the experiments described below, the average temperature ranged from 24.7° C. to 26.7° C., with a maximum variation within each experiment of 0.2° C.

Eggs heavily poisoned (8.32 mM/L) 44–53 minutes before control 50 per cent cleavage, *i.e.,* early prophase, maintained a high percentage of intact nuclei, although the number fluctuated as if the nuclei were dissolved and reforming. In experiment III the first drop in numbers of nuclei was preceded by a period of persistent asters. The second decline, at 13 hours, showed no evidence of mitotic activity other than the disappearance of half of the nuclei—which then reappeared by 22 hours. At slightly lower doses (6.25 and 4.16 mM/L) an appreciable number of eggs divided, preceded by a normal dissolution of the nucleus. But nuclei also disappeared about the same time in 25–75 per cent of the eggs which did not divide. Moreover nuclei reappeared in only 25–75 per cent of the divided eggs. Hughes (1950) also reports reconstruction of nuclei in cells blocked at metaphase by nitrogen mustard.

Exposure begun when the eggs were in late prophase or in metaphase retarded the first cleavage, but permitted most of the eggs to divide. At 8.32, 6.25 and 4.16 mM/L the effects were the same as with doses earlier in the cycle, except that they appeared one cleavage later. Nuclei appeared in some of the blastomeres, and some of these then disappeared, usually coincident with cleavage. Most of the blastomeres resulting from the second and later cleavages did not form nuclei. A single nucleus appeared in about half of the eggs which did not divide. A small percentage of the one-cell and two-cell stages formed karyomeres instead of whole nuclei. Occasionally a blastomere became binucleate or underwent multiple cleavage.

Exposure to 6.25 mM/L at the end of the first mitotic cycle or early in the second produced the same phenomena one stage later. Nuclei appeared in most of the blastomeres. Half of them disappeared at a time when other eggs were fragmenting, and in experiment VI, at least (exposure 17 minutes before 50 per cent second cleavage), most of these nuclei had reappeared by 9 hours after fertilization. At the two-cell and four-cell stages, karyomeres appeared in from 6 to 100 per cent of the blastomeres, depending upon the experiment. Rarely a blastomere at the two-cell stage was binucleate. Exposure begun when some of the eggs had already divided twice yielded a nuclear cycle in the four-cell stage: nuclei reaching 64 per cent, then dropping to 34 per cent as some of the eggs divided, and finally disappearing from all eggs at 9½ hours, when a fourth of the eggs were disintegrating.

These data show that even when eggs are exposed in early prophase, and poisoned to the extent that the prophase is much prolonged, the nucleus nevertheless dissolves and often re-forms. These reformed nuclei may be the "prophases" observed by others. Exposure late in the mitotic cycle shows that eggs can be stopped after completion of the prophase, presumably at metaphase since subsequently many form a single nucleus. Karyomeres, binucleation and multiple cleavage testify to the varying degrees of disruption to which the telophase is subject.

If there were a prophase effect such as is found in the intact mammal, one would expect that at the time for the first prophase, or at least at the second prophase, the nucleus would fold up into a pycnotic mass and the whole cell would expire. Yet, the prophases appear in an orderly manner, the asters build up, and a spindle replaces the nucleus. Such a mitotic figure might persist for an hour and a half, and eventually succeed in dividing the egg. Indeed, if any one phase is to be considered sensitive in the sea-urchin egg, it is telophase. Frequently the eggs almost divide and then the furrow regresses, leaving a binucleate egg.

Evidence for prophase damage by mustards comes mostly from experiments with intact animals (Dustin, 1947; Friedenwald and Buschke, 1948; Gillette and Bodenstein, 1946). It is not found in isolated cells such as Arbacia eggs (Hutchens and Podolsky, 1948) or tissue cultures (Hughes, 1950; Meier and Schär, 1947; Fell and Allsopp, 1949) or in some plants (Novick and Sparrow, 1950; Hohl, 1948). This suggests that systemic influences on mitosis be considered before different sensitivities of division phases be ascribed entirely to intrinsic properties of the mitotic cycle.

Synergism with urethan

Skipper (1949) has found a synergism between nitrogen mustards and urethan in decreasing the white cell count in mice. For comparison, eggs were exposed to 22.5 and 45 mM urethan, and 0.032 and 0.065 mM HN2 (No. 5) separately or combined.

Urethan alone at 22.5 mM retarded first cleavage 6 minutes, and second cleavage 6 minutes and retarded the gastrulae. HN2 alone at 0.032 mM retarded the second cleavage 1½ minutes and caused abnormal blastulae. Together they retarded the first cleavage one-half minute and the second cleavage three minutes and produced abnormal blastulae.

In urethan alone at 45 mM, only 12 per cent of the eggs had divided an hour after the controls divided, and the gastrulae were irregular. HN2 at 0.065 mM did not affect the first cleavage, but retarded the second 7 minutes, and produced abnormal blastulae. These doses combined gave 12 per cent cleavage an hour late, and irregular blastulae.

The effects are no more than the separate effects of the urethan on the early cleavages and HN2 on the larvae. The synergism observed in mice would appear to involve systemic effects which do not operate on isolated dividing cells. Further, the different patterns of effect and of dose-effect ratios indicate caution (already pointed out by Loveless and Revell, 1949) in grouping these poisons together as "radiomimetic" (Dustin, 1947).

SUMMARY

1. The capacities of different mustards for blocking or delaying the cleavage of sea urchin eggs were compared by exposing the eggs of *Arbacia punctulata, Tripneustes esculentus* and *Lytechinus variegatus* to freshly prepared solutions continuously, beginning 10 to 13 minutes after fertilization.

2. At least two β-halo groups are necessary to confer the highest potency of the molecule. A third β-chloroethyl or a second *bis*-(β-chloroethyl)-amine group, or a non-chlorinated substitution on the nitrogen alters the activity of the molecule. β-chloropropyl or γ-chloropropyl can substitute for the β-chloroethyl radical. Methyl *bis*-β-bromine was slightly more active than its chlorine homologue and a fluorine congener was probably less active.

3. Environmental conditions have not been standardized in these experiments to permit an evaluation of differences in susceptibility of the different species.

4. In the range of 0.065–1.040 mM methyl-*bis*-(β-chloroethyl)-amine, retardation of second cleavage increases slowly with successive doublings of dose. Increasing the concentration beyond 1.040 mM rapidly decreases the percentage of eggs which divide.

5. The course of events in eggs exposed at the beginning of prophase, and later in the first and subsequent mitotic cycles points to a general slowing of mitosis. Any phase of mitosis can be blocked, depending upon the dose and time of exposure.

6. There is no synergism between urethan and methyl-*bis*-(β-chloroethyl)-amine when they are combined in threshold doses.

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STUDIES ON THE ACROSOME. I. REACTION TO EGG-WATER AND OTHER STIMULI[1]

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Our knowledge of spermatozoa and their role in the fertilization reaction has been acquired through studies approaching the subject from two widely different angles—"straight" morphological observation dealing with fixed specimens, such as the classical drawings of Retzius, or the electron microscope photographs of Bretschneider; and physiological studies centering around the fertilizin theory of F. R. Lillie and currently being pushed close to conclusion by the penetrating biochemical research of Tyler, Hartmann, Runnström and their co-workers.

One previous attempt to correlate the morphological and physiological aspects of sperm agglutination has been presented in the paper of Rötheli, Roth and Medem (1950), which deals with several species of freshwater fishes. The reaction in these forms, however, is an irreversible one, in which the spermatozoa become more and more firmly aggregated into increasingly dense clumps, and it seems difficult to distinguish possible normal changes in form due to agglutination from those accompanying the death of the spermatozoa.

In sea urchins, because of the small size and violent activity of spermatozoa in their normal state, work on living sperm has almost necessarily dealt with them in bulk suspensions; the vital staining study by Popa in 1927 is a rare example of an effort to examine living spermatozoa one at a time. A tendency in this direction is also to be found in Carter's 1931 paper, in which he stresses the fact that considerable physiological differences can be discerned between the gametes of a single induced shedding.

With the development of the phase contrast microscope, it has become possible to distinguish hitherto invisible details of active spermatozoa in a sufficiently normal state that observations can be made of their behavior under various conditions (Dan, 1950). Phase microscopy also provides a stepping-stone to the use of the electron microscope, by permitting a large degree of comparison between the living state and the electron photographs, and to a great extent obviating the uncertainty concerning the appearance of artifacts which would otherwise inevitably attach to a method involving observation *in vacuo*. All the points discussed in this paper have been checked in the living condition and in spermatozoa immediately after fixation in sea water suspension, and it can be stated with confidence that no qualitative difference was found among the three types of observation.

MATERIALS AND METHODS

The spermatozoa photographed in this study belong to the two sea urchin species, *Pseudocentrotus depressus* and *Strongylocentrotus pulcherrimus,* although

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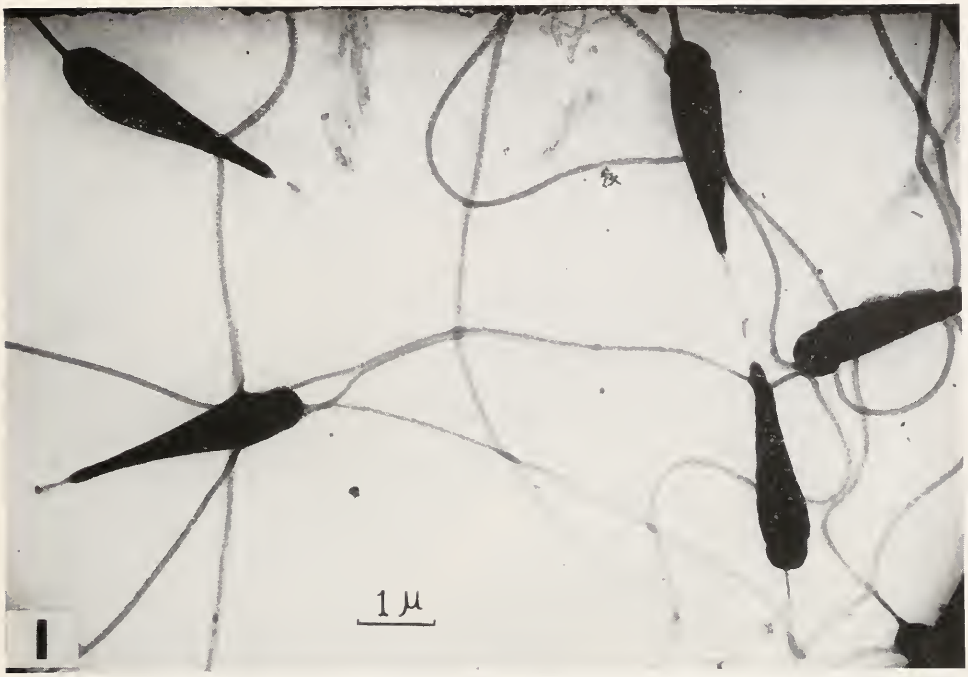

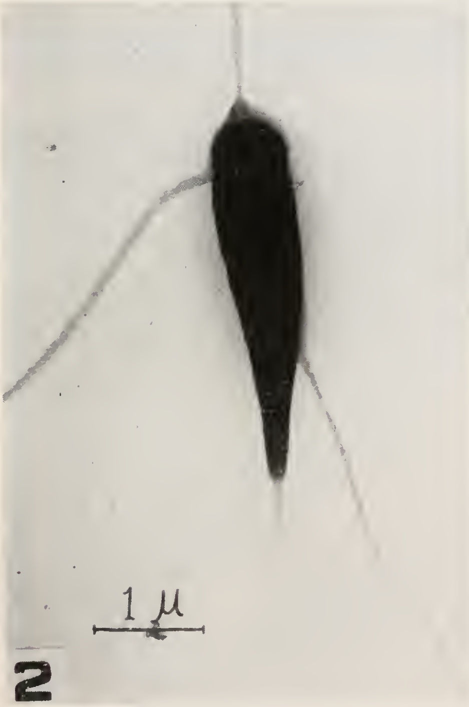

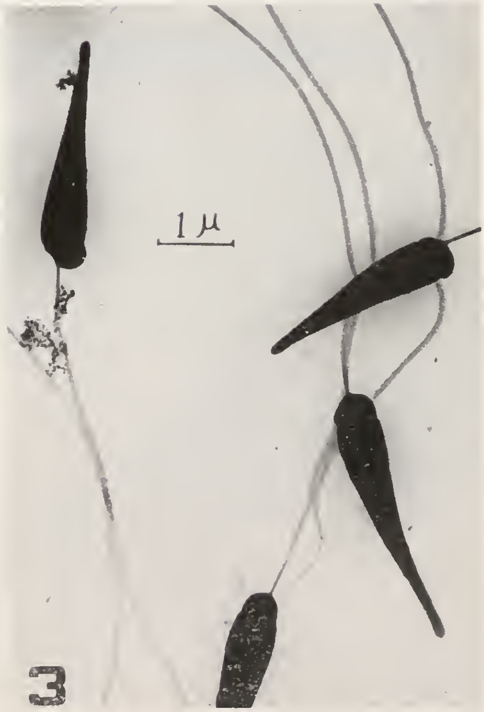

FIGURE 1. Spermatozoa of *Pseudocentrotus depressus*, fixed 30 seconds after the addition of egg-water.

FIGURE 2. Same as Figure 1.

FIGURE 3. Control in ordinary sea water.

phase contrast observation has shown that the phenomenon in question is generally characteristic of the other echinoids available at Misaki.

In every case, suspensions were made from dry sperm secured by removal of the testes with care to avoid contamination by sea water or body fluid; only freshly suspended spermatozoa were used. Egg-water was obtained by inducing shedding of eggs into filtered sea water (by electrical stimulation of intact females in the case of Pseudocentrotus (see Iwata, 1950), and by introducing a few drops of isotonic KCl into the cut test of Strongylocentrotus); the suspension was stirred occasionally during a minimum of two hours, and the titer of the supernatant jelly-sea water solution roughly determined by low-power microscopic observation of the intensity and duration of the agglutination reaction which occurred when a given amount of this egg-water was mixed with the standard sperm suspension (ca. 0.025 cc. dry sperm in 2 cc. sea water).

Irreversible aggregation of the spermatozoa was induced by suspending them directly in sea water of pH 9.2. For the first 30 seconds no heterogeneity could be detected in the distribution; from 40 seconds a tendency to clumping was apparent; by two minutes most of the spermatozoa were involved in aggregations approximately the size of those formed at the height of the reversible agglutination reaction. The suspension was fixed at this stage.

The spermatozoa were fixed by the addition of neutralized formalin to the suspensions; they were left for at least 24 hours in this solution before dilution of the sea water. The change to 5–7% formalin in distilled water was carried out by allowing the suspended spermatozoa to settle naturally, since it was found that even gentle centrifuging caused clumping.

Electron photographs were taken with a Hitachi standard type electron microscope, at an operating voltage of 50 KV. Shadowing was done with Cr_2O_3.

RESULTS

In both species, the agglutination reaction occurs instantanously upon addition of egg-water to a sperm suspension; at the titer chosen as standard, the clumping of spermatozoa is at its maximum—*i.e.*, the greatest numbers of spermatozoa are involved in the clumps—at about 30 seconds after addition of the egg-water, and dispersal is complete within 2½–4 minutes. Figures 1 and 2 show spermatozoa fixed at the height of the agglutination reaction; Figure 3 is a photograph of spermatozoa similiarly suspended and fixed 30 seconds after the addition of the same amount of plain sea water. It is clear that a change has occurred in the region of the acrosome, under the influence of the egg-water; the probable nature of this change will be discussed below.

In Figures 4 and 5 are shown two photographs of Pseudocentrotus spermatozoa fixed 5 seconds after the addition of egg-water. It is important that in spermatozoa thus fixed immediately after reaction to the egg-water stimulus, the acrosome sub-

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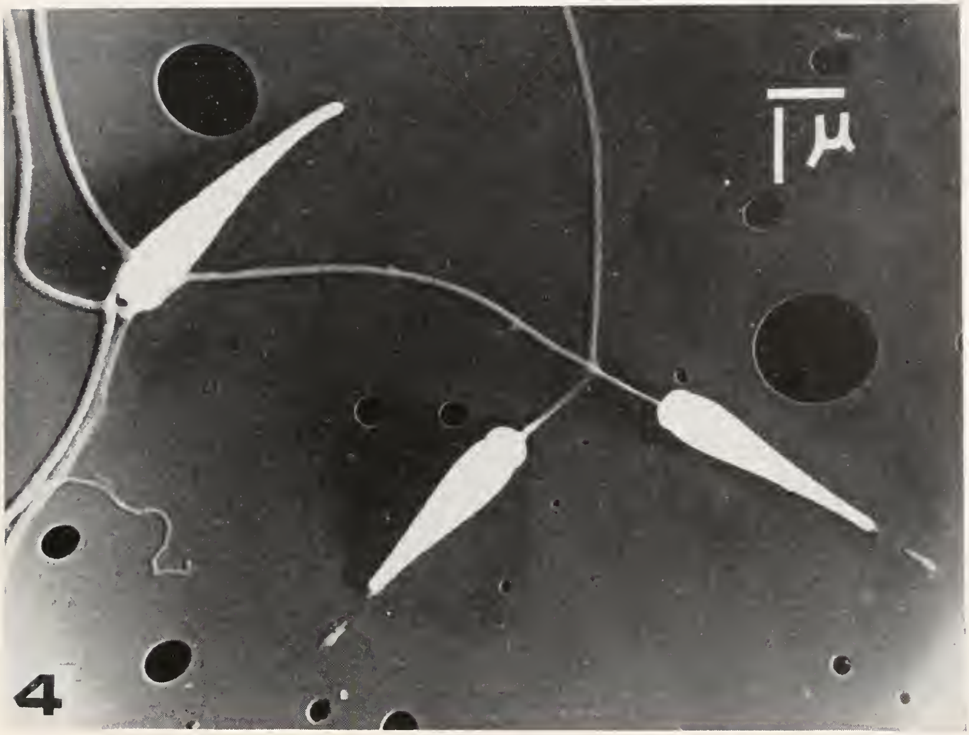
FIGURE 4. Pseudocentrotus spermatozoa fixed 5 seconds after addition of egg-water. The spermatozoan at the upper left has failed to react. The breakage between the head of the spermatozoan and the extruded acrosome substance is caused by drying on the collodion membrane. Shadowed with Cr_2O_3, $\tan^{-1} = \frac{1}{2}$.

FIGURE 5. Same as Figure 4, magnification 32,000 ×.



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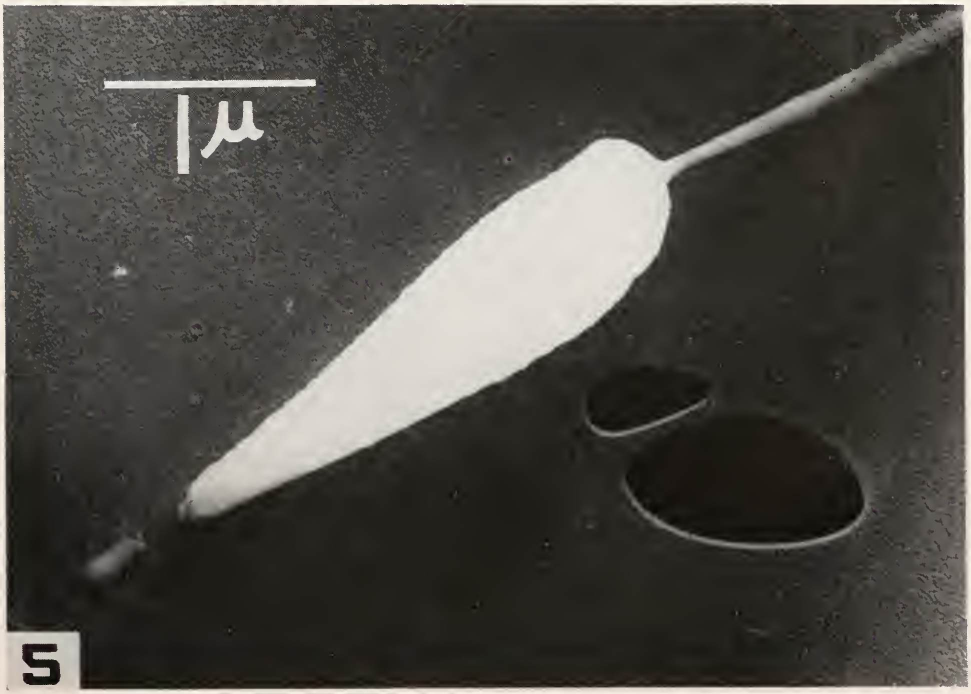

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stance appears as a drop-like mass on the tip of the head. The break which almost invariably occurs between the tip of the sperm and the substance attached to it in such early fixation is interpreted as an artifact due to the contraction of the preparation on drying. Comparison of these photographs with Figures 6 and 7, of spermatozoa fixed at 2 minutes 35 seconds after reversal of agglutination, gives the impression that part of the acrosome substance disperses to a considerable extent, while a central core or fiber of some sort (which may be a separate structure that appears secondarily), possessing greater cohesiveness and not dispersing readily in sea water, remains attached to the former acrosomal region.

Figures 8 and 9 show photographs of Strongylocentrotus spermatozoa, fixed 25 seconds after addition of egg-water and plain sea water, respectively, to fresh suspensions. Figure 10, of spermatozoa fixed after two minutes in sea water at pH 9.2 indicates that morphologically the same sort of reaction is called forth by alkalinity as by egg-water.

Figures 11 through 15 are photographs of spermatozoa which were placed in living suspension (in plain sea water) directly onto a collodion membrane mounted for electron microscope observation, in order to test the possibility that the reaction of the acrosome may be induced by simple contact with solid objects, in the absence of egg jelly substance. The sperm were observed with low power of an ordinary microscope to ascertain that most of them were attached to the membrane by their tips and rotating. A drop of formalin-sea water was then added, at 105 seconds after suspension, and changed several times to remove unattached spermatozoa; after three hours fixation in formalin-sea water, the preparation was washed with dilute formalin and then distilled water before desiccation.

The photographs show that attachment to the collodion membrane induces a reaction essentially the same as that produced by egg-water. The acrosome substance is set free, appearing as an amorphous mass which fails to form a clear boundary in sea water and tends to spread on the collodion surface.

Discussion

Popa, after staining living *Arbacia punctulata* spermatozoa with Janus green, reports (p. 251), ". . . one gets the impression that the spermatozoa eliminates through the point of the head (where really there is an exceedingly minute opening) very small amounts of an extremely sticky substance." In egg-water (p. 256), "This appears as a small granule on the points of almost all spermatozoa. The adhesion of the spermatozoa to one another or to other objects is made by means of this granule."

With phase contrast oil immersion, it is easy to recognize Popa's "small granule" at the tip of the acrosome of unstained spermatozoa freshly suspended in sea water, of several sea urchin species. It is also just barely possible to make out a small mass of colorless, non-refringent substance attached to the tips of spermatozoa in egg-water (after reversal of agglutination, or immediately after addition of the egg-water if this is done under a cover-glass so that the spermatozoa become attached

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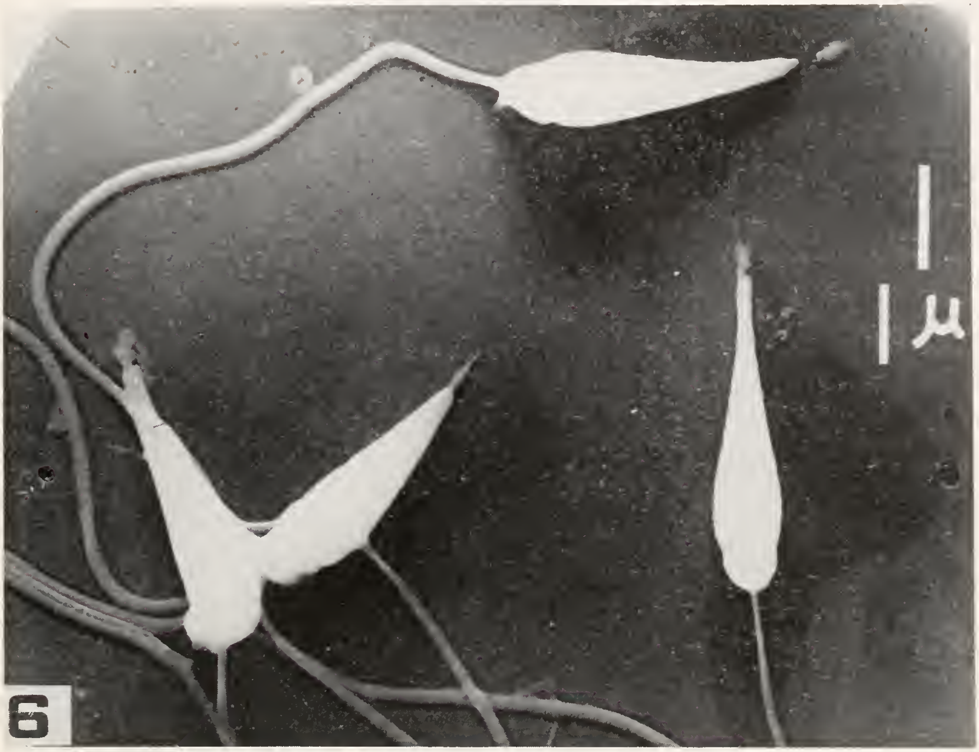
Figures 6 and 7. Pseudocentrotus sperm fixed after reversal of agglutination (2 minutes 35 seconds after addition of egg-water).



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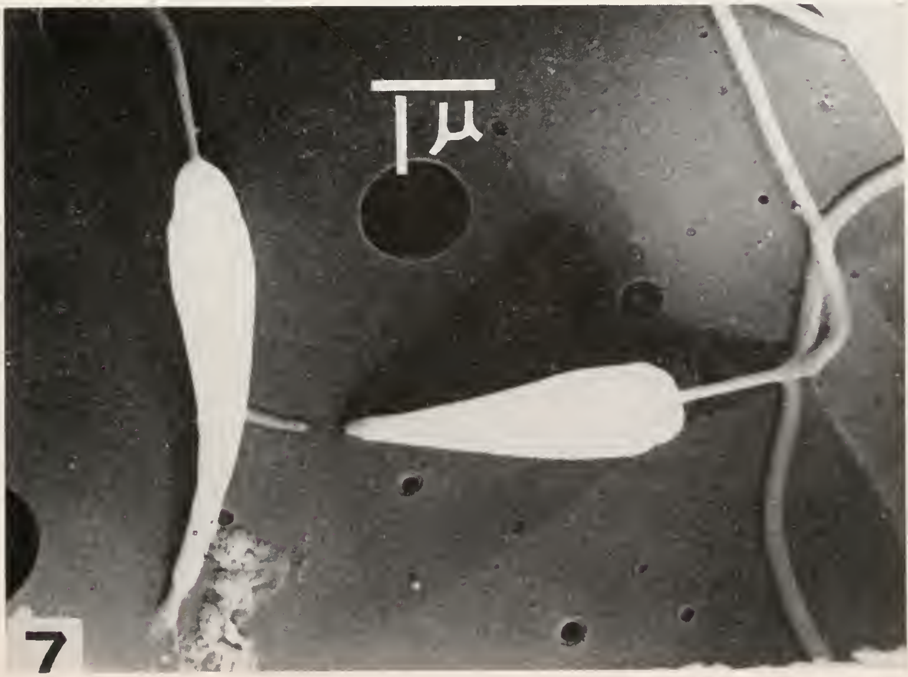

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to the glass surfaces instead of forming clumps). When such spermatozoa are freely suspended, the substance appears as a flabby, gelatinous "tongue" of uniform diameter (roughly 0.2 μ), which may reach a length of more than 1 μ and is always in vibratory, Brownian-like motion. That it is exceptionally sticky in normal sea water is attested to by the fact that once this substance has come into contact with any surface, it is never pulled loose even by the most vigorous movements of the spermatozoan. A few cases have been observed in which active spermatozoa have broken away, leaving at least part of the "tethering" substance on the glass surface, but it is much more common to find them permanently attached by the highly pliable strand which obviously possesses considerable cohesiveness.

As for Popa's "minute opening," however, it is probable that what he was seeing was rather a denser portion at the extreme tip of the acrosome, which appears darker than the more proximal part with phase contrast.

Before this study reached the stage of electron microscopy, it seemed fairly apparent that a gelatinous substance was extruded from the tip of the acrosome in response to the stimulus of egg-water, alkaline sea water and contact with solid surfaces. However, careful study of all the electron photographs seems to indicate rather that the anterior part of the acrosome undergoes a drastic change within one or two seconds after such stimulation. This is most clearly shown in Figure 4, in which the one spermatozoan which has failed to respond immediately to the egg-water stimulus is long and tapering at the apex, while the two others which show the typical reaction are truncated.

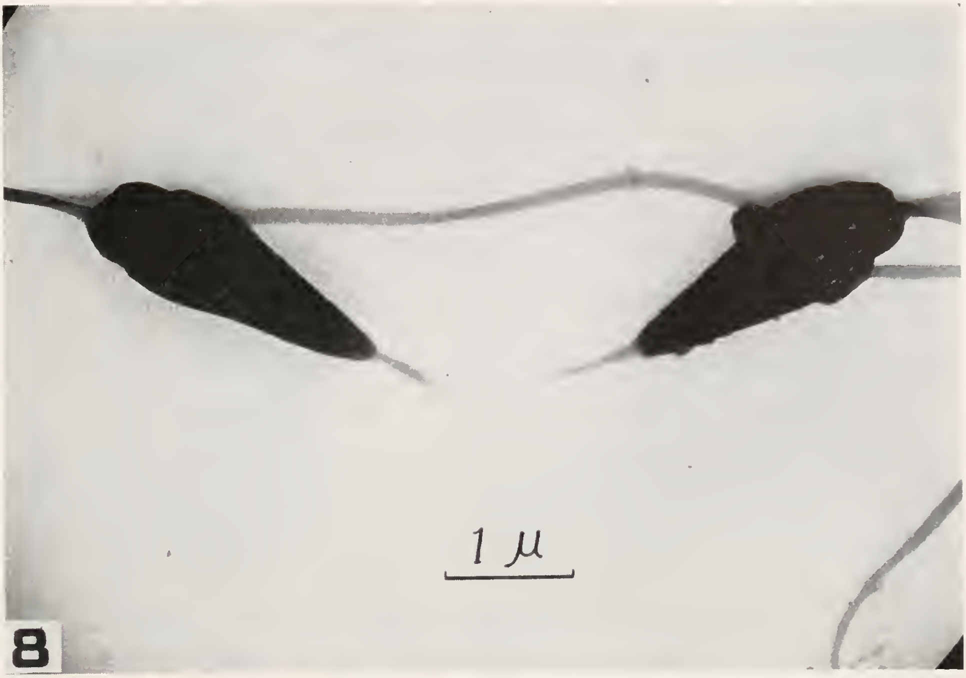
It is proposed that the condition of the acrosomal region in sperm which have so reacted (*cf.* Fig. 5) can best be explained by imagining that the plasma membrane investing the tip of the acrosome undergoes local autolysis, leaving the underlying substance exposed. If this substance is, or includes, an *egg-membrane* lysin such as that isolated by Tyler (1939) and Krauss (1950a, 1950b), or an *egg-surface* lysin like Androgamone III of Runnström *et al.* (1944, 1945, 1946), the postulated breakdown of the acrosome membrane would leave the lysin free to attack the vitelline membrane of the egg as soon as the spermatozoan carried it into contact with the egg surface.

The two widely differing lytic substances identified by these two groups of workers are obtained from frozen-thawed or frozen-dried sperm; there has so far been presented no unequivocal evidence as to their ready availability or exact location in the living spermatozoan, although Tyler (1949) has published electron micrographs of keyhole limpet spermatozoa showing that extraction with alkaline sea water (pH 9), which yields a solution of the egg membrane lysin, causes a more or less drastic breakdown of the acrosome. Pending tests of its chemical properties and lytic activity, it can only be offered as a surmise that the acrosome substance under discussion in this paper contains either or both of the lysins identified by Tyler and Runnström.

FIGURE 8. Strongylocentrotus spermatozoa fixed 30 seconds after agglutination with egg-water.

FIGURE 9. Strongylocentrotus sperm fixed 30 seconds after addition of plain sea water to suspension (control).

FIGURE 10. Strongylocentrotus sperm fixed after 2 minutes in sea water of pH 9.2.



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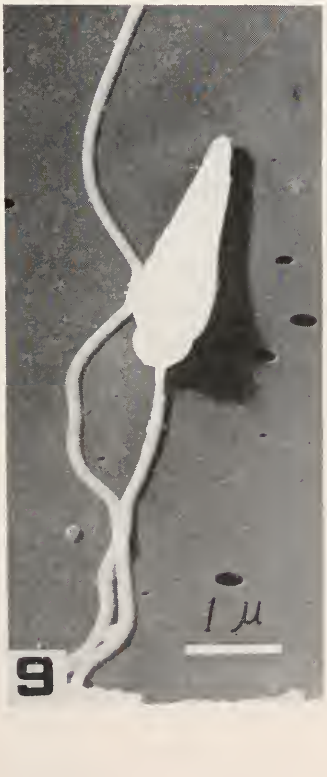

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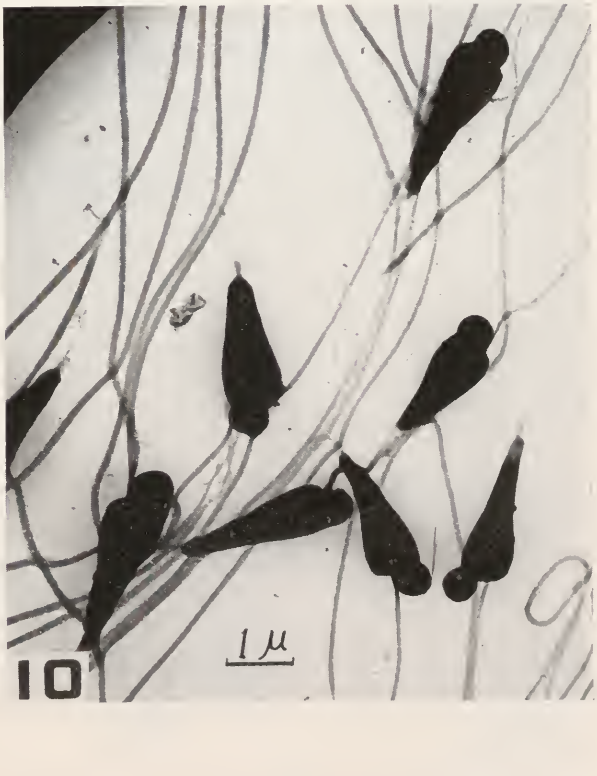

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In support of that surmise, certain circumstantial evidence can be adduced. The time relationships satisfactorily fit the requirements of the process as it must occur in normal fertilization, if it is assumed that the acrosome membrane is stimulated by the jelly substance, either intact or in solution in the immediate vicinity of the egg, and reacts during the very few seconds which are required for the spermatozoan to pass through the jelly layer and reach the vitelline membrane.

The fact that this reaction of the acrosome is also induced by hyperalkalinity in the absence of the species specific factor contained in the egg jelly would account for the long-known empirical fact that raising the pH of the medium facilitates sperm penetration in inter-species crosses, self-fertilization in self-sterile hermaphrodites, and other cases in which fertilization is blocked in normal sea water.

Furthermore, the observed reaction to contact with solid objects offers a fairly satisfactory explanation for the fact that removal of virtually all the jelly from unfertilized eggs by acid extraction does not prevent fertilization.

On the basis of these various observations, it is proposed that there are two separate phenomena simultaneously involved in egg-water agglutination of sea urchin sperm: a specific reaction (fertilizin-antifertilizin) which orients the spermatozoa into regular groups and holds them so for a definite period; and a response of the acrosome to the chemical stimulation of the dissolved jelly, which is manifested by an almost instantaneous local breakdown of the acrosome membrane so that the acrosome substance is exposed at the tip of the sperm head as a relatively labile mass. This response of the acrosome is believed to be less specific in nature than the agglutination reaction, being induced also by hyperalkalinity and (possibly somewhat less readily) by contact with solid surfaces.

Applying this experimental conclusion to natural fertilization, it is suggested that as the spermatozoan actively swims through the loose network of the jelly layer, it responds to the chemical stimulation of the jelly substance by a breakdown of the membrane covering the front part of the acrosome, so that by the time the sperm reaches the egg surface, a few seconds later, it carries at its tip a mass of freshly exposed lysin with which it effects penetration of the vitelline membrane as the first step in the fertilization process.

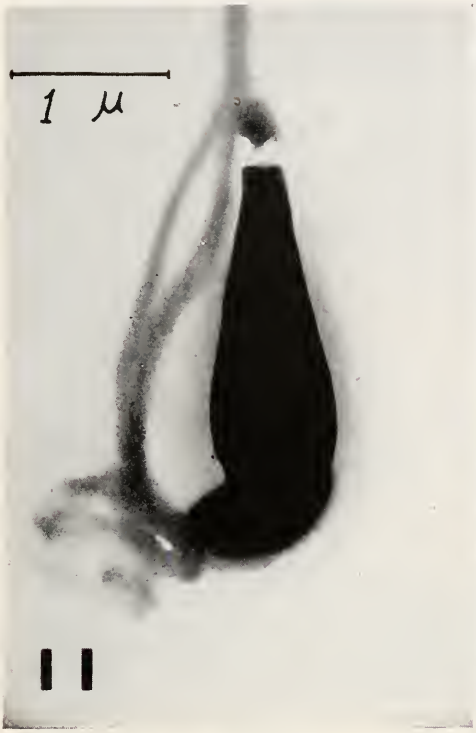
In a discussion of the properties of fertilizin, Tyler (1949) makes the following statement (p. 203): ". . . experiments show that fertilizin acts as an aid to fertilization, but only when it is present on the surface of the egg, as the gelatinous coat. . . . Evidently when the interaction of the egg and fertilizin takes place away from the egg and is completed before the sperm reaches the surface of the egg, the sperm are then unable to combine with the egg." Assuming that the sea water-labile portion of the acrosome substance is a vitelline membrane lysin, its demonstrated dispersal within less than 30 seconds after contact with egg-water (fertilizin)

FIGURES 11–13. Spermatozoa of *S. pulcherrimus;* freshly suspended sperm fixed on collodion membrane, showing various degrees of acrosome reaction.

FIGURE 11. Acrosome partially reacting to stimulus of its own tail.

FIGURE 12. Acrosome of sperm at right completely reacted to contact with collodion membrane.

FIGURE 13. These three spermatozoa were rotating about their tips on the collodion membrane when fixed. (This photograph was intentionally under-exposed to show differences in density of acrosome region. Fully exposed print shows acrosome substance spread on collodion membrane around points of attachment, similar to condition in Figure 15.)



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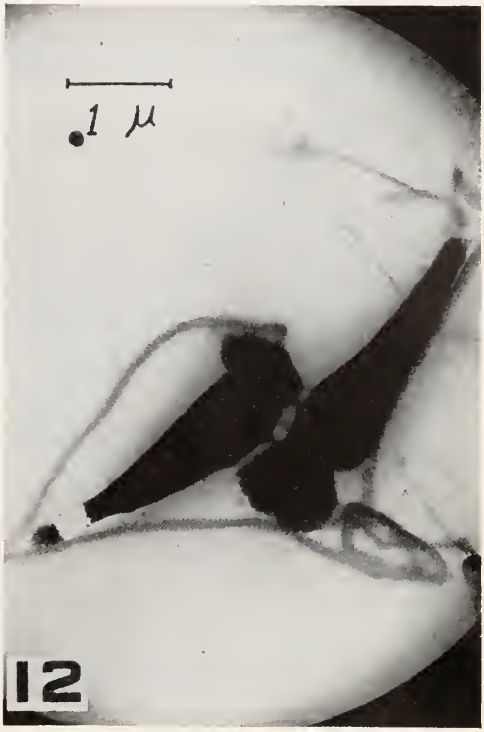

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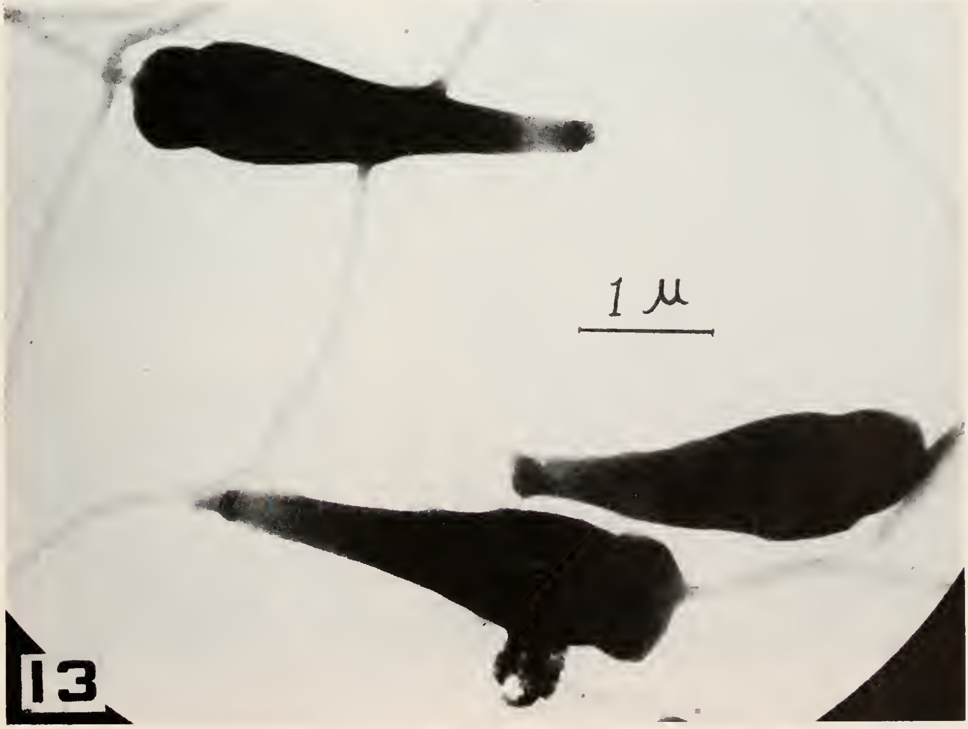

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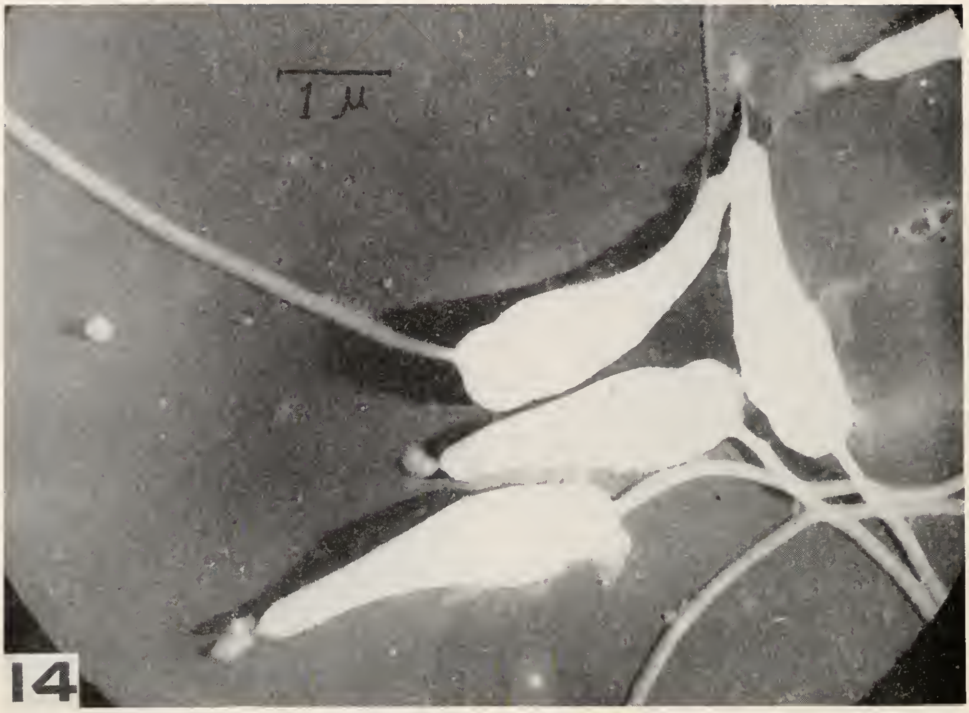

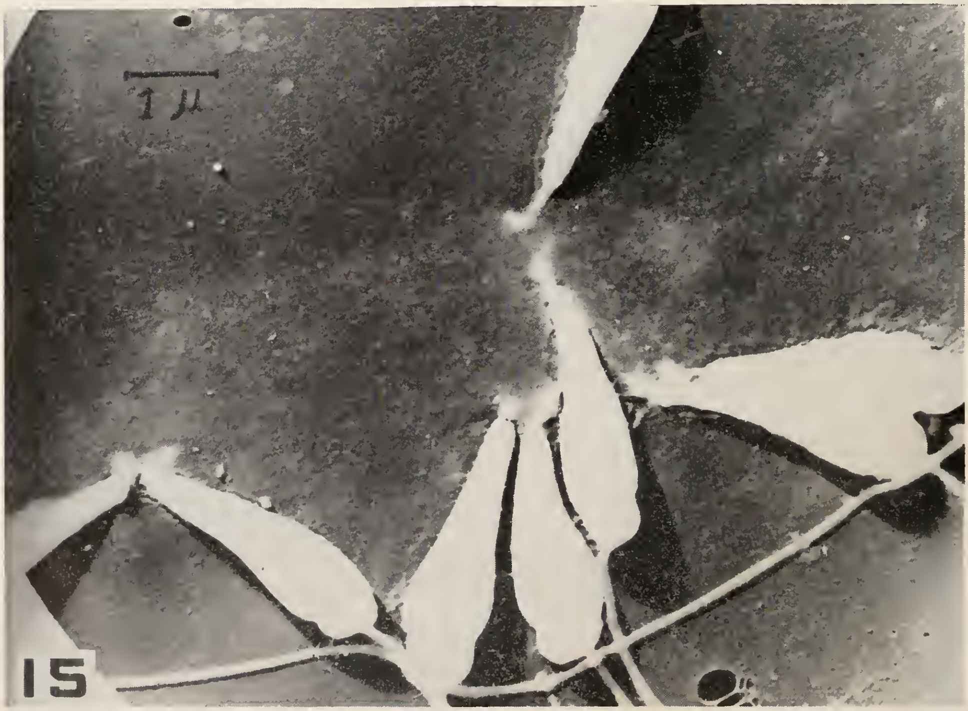

Figures 14 and 15. Spermatozoa fixed while rotating around tips on collodion membrane. Acrosome substance newly exposed (Fig. 14) and in several degrees of spreading on collodion surface (Fig. 15). Shadowing with Cr_2O_3; $\tan^{-1} = \frac{1}{3}$.

would seem to furnish an adequate explanation for the subsequent failure of the spermatozoan to effect penetration of the egg.

Krauss (1950a) has worked out in great detail the conditions under which egg-membrane lysin is obtainable from spermatozoa of the keyhole limpet. One of his most striking cases, that of increased pH, coincides with the result reported in this paper, and there is nothing in his other observations which is contrary to the suggestions made here. Coordination of the experimental conditions might very well bring out an interesting positive agreement between the two sets of results.

The writer wishes to express sincerest gratitude to Prof. A. Takamiya, of Tokyo Institute of Technology, for his enthusiastic encouragement and kindness in arranging for the use of the Institute's electron microscope; and to Mr. H. Akabori, of the Institute, who has cooperated most generously in preparing and photographing the material. The writer's thanks are also due in large measure to the Director and Staff of the Misaki Marine Biological Station for their unfailing courtesy in sharing the Station's facilities; and finally to Drs. C. B. Metz, D. Mazia and K. Dan, for their exceedingly helpful criticism of the manuscript.

SUMMARY

1. Spermatozoa of two sea urchin species were fixed with formalin in sea water, egg-water and sea water of pH 9.2, and observations of fixed specimens made with phase contrast and electron microscopes were compared with phase contrast observation in the living state.

2. In response to egg-water, a reaction is called forth which is interpreted as a breakdown of the acrosomal membrane, by which a labile mass of substance is exposed at the tip of the spermatozoan head.

3. This same reaction is induced by exposure to sea water of pH 9.2, and by contact with solid surfaces.

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THE INFLUENCE OF TEMPERATURE ON SYNAPSIS IN HYBRID SALIVARY GLANDS[1]

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The giant chromosomes of Diptera are the most favorable material for studies of the extent and nature of synaptic association. The mitotic and meiotic chromosomes in this group are so small that they hardly offer a clear picture of the details of their conjugation during prophase or interphase. It has become customary, therefore, to rely on salivary glands for studies of synaptic association in structural hybrids of Drosophila, although it is not known whether the synapsis of giant chromosomes reflects with any degree of accuracy the mode of association of homologues in meiosis or mitosis. While the conjugation of homologues is believed to be a prerequisite for the occurrence of crossing-over, the observed variations in cross-over frequency and their relationship to the phenomenon of synapsis are not understood. It seems desirable, therefore, to determine whether synaptic association varies with environmental factors, which are known to influence the frequency of crossing-over. Beginning with Plough (1917), much attention has been paid to the effect of temperature on crossing-over. It was for this reason that temperature was chosen as the varying factor in the present study.

The question of variation in synapsis, apart from its possible bearing on crossing-over, gains additional interest in the light of the "Structural Theory" of the position effect mechanism (Ephrussi and Sutton, 1944; Gersh and Ephrussi, 1946).

Variation in the extent of synapsis with age and temperature was observed in salivary glands of Chironomus hybrids (Goldschmidt, 1947). Synapsis was found to decrease with rising age of the larvae. In larvae grown at high (30° C.) and low (13° C.) temperatures, there was less synapsis than in larvae reared at moderate temperatures (20–25° C.). While salivary glands of Chironomus offer material for smears during an extended prepupal period, the suitable stage for smearing in Drosophila is of very short duration. This precise definition of the physiological age suitable for smearing should tend to minimize any possible age effect on synapsis in preparations obtained from this organism and should render them a suitable object for the study of the temperature effect.

Materials and Methods

The effect of temperature on synapsis was examined in two structural hybrids in the genus Drosophila. As an instance of a small inversion within a species, the

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[1] This work was carried out in the Zoology Department, University of California, Berkeley, during the tenure of the Mary E. Woolley International Fellowship of the American Association of University Women, awarded by the International Federation of University Women.

I am greatly obliged to Dr. C. Stern for his generous help and advice, and to Dr. Aloha M. Hannah for many valuable suggestions.

Arrowhead inversion in Chromosome III of *D. pseudoobscura* was chosen. For a large inversion in a species hybrid, the condition of Chromosome III R in the *D. melanogaster* × *simulans* hybrid was studied.

For the *D. pseudoobscura* crosses, 10 females and 10 males were mated in half-pint bottles and allowed to oviposit for three days at room temperature. The parents were then removed and the bottles transferred to constant temperature chambers of 13, 17.5, 20, 25 and 28° C. (this being the upper limit for larval development in this species).

The *D. melanogaster* × *simulans* cross was obtained by the method described by Uphoff (1949), 10 *melanogaster* females and 15 *simulans* males being mated (without etherizing) in "creamers." If the cross was found to be fertile, the parents were removed to half-pint bottles and allowed to oviposit for two days, at room temperature. After removal of the parents, the bottles were kept at 13, 17.5, 20, 25, 29 and 31° C. Although the food employed was molasses-cornmeal-agar enriched with yeast, "yeasting" of the bottles at the time of appearance of second instar larvae was found indispensable for obtaining good preparations of salivary chromosomes.

The glands were dissected, at room temperature, in 45% acetic acid, stained in aceto-carmine and made permanent according to a method of Dr. A. M. Hannah, by transferring through alcohol and xylol into Canada Balsam, without removing the cover-slip, whenever possible.

The preparations examined for the *pseudoobscura* cross include male and female offspring of reciprocal matings between Standard and Arrowhead. The *melanogaster* × *simulans* preparations were all from female larvae of the cross employing *melanogaster* as the female parent.

The following arbitrary system of recording asynapsis was adopted: *D. pseudoobscura*. The loop extends from band 70B to 76B in Chromosome III (Dobzhansky, 1944).

	Asynaptic units
Loop completely unsynapsed	3
Loop synapsed except for sections 70B–D and/or 76A–B	1
Loop synapsed less than this	2
Complete asynapsis in section before loop (63A–70A)	1
Any degree of asynapsis in section before loop	½
Complete asynapsis in section after loop (76C–81D)	1
Any degree of asynapsis in section after loop	½

D. melanogaster × *simulans*. The loop (figured by Pätau, 1936) extends from band 84F to 93E.

	Asynaptic units
Loop completely unsynapsed	4
Loop three-quarters unsynapsed	3
Loop half unsynapsed	2
Loop one-quarter unsynapsed	1
Loop less than one-quarter unsynapsed	½
Section before loop (81F–84F) completely unsynapsed	1
Section before loop partly unsynapsed	½
Section after loop (93F–100F) completely unsynapsed	3
Section after loop two-thirds unsynapsed	2
Section after loop one-third unsynapsed	1
Section after loop less than one-third unsynapsed	½

In order to avoid unconscious selection, the labels of all preparations were covered with non-transparent paper before scoring. Twenty nuclei were scored in each preparation of the *pseudoobscura* cross and ten nuclei in each of the *melanogaster* × *simulans* crosses.

RESULTS

a) *Arrowhead* × *Standard inversion*

The results are presented in Table I and Figure 1. There was more asynapsis at 13° C. on one hand and at 28° C. on the other than at intermediate temperatures (17.5–25° C). Although the standard errors are large, the difference between 13° C. and 17.5° C. and the difference between 25° C. and 28° C. are significant. The slight rise in asynapsis between 17.5° C. and 20° C. and the decline from 20° C. to 25° C. are not significant. It may be concluded that synapsis in this inversion is comparatively most complete at intermediate temperatures. The large standard errors reflect, of course, the considerable differences existing between different individuals grown at the same temperature. Moreover, as is known to every worker with salivary gland chromosomes, the variation between nuclei of the same gland is also pronounced. Nevertheless, a cursory inspection of a preparation was usually sufficient to determine whether the larva had grown at an extreme or at an intermediate temperature.

TABLE I

Asynaptic units in Arrowhead-Standard inversion

Temp. °C.	No. of larvae	Mean asynaptic units	Standard error	t	p
13	15	30.2	±7.3		
				3.2	0.01–0.001
17.5	14	22.5	±5.8		
				0.7	0.6 –0.5
20	13	23.7	±3.2		
				1.3	0.3 –0.2
25	14	21.3	±6.0		
				4.3	<0.001
28	13	30.0	±4.3		

b) *Melanogaster* × *simulans inversion*

The results are summarized in Table II and Figure 1. Synapsis is most complete at 13° C. There is less pairing at 17.5° C. Asynapsis falls off from 17.5° C. to 25° C. and then rises again until 31° C. The differences between 13° C. and 17.5° C., 17.5° C. and 25° C. and between 25° C. and 31° C. are significant.

It should be mentioned that the preparations of the 13° C. series could usually be assigned to this group without reading the label. Although they do not show significantly more synapsis than those of the 25° C. group, their chromosomes are peculiar, because the fusion of the homologues in synapsed regions is usually less intimate than in any other series. Since under the present system of scoring any mode of contact between homologues was recorded as "synapsis," the particularly loose synapsis of the 13° C. group is not expressed in Table II.

TABLE II

Asynaptic units in melanogaster × simulans inversion

Temp. °C.	No. of larvae	Mean asynaptic units	Standard error	t	p
13	22	15.4	±5.2		
				3.3	0.01–0.001
17.5	15	22.6	±8.0		
				0.7	0.5 –0.4
20	13	20.5	±7.2		
				1.4	0.2 –0.1
25	17	17.3	±5.3		
				1.4	0.2 –0.1
29	17	19.6	±4.5		
				2.4	0.05 –0.02
31	10	23.8	±4.3		
Significance of difference between:					
Temperatures ° C.					
13 and 25				1.1	0.3 –0.2
17.5 and 25				2.2	0.05–0.02
25 and 31				3.3	0.01–0.001
13 and 31				4.5	<0.001

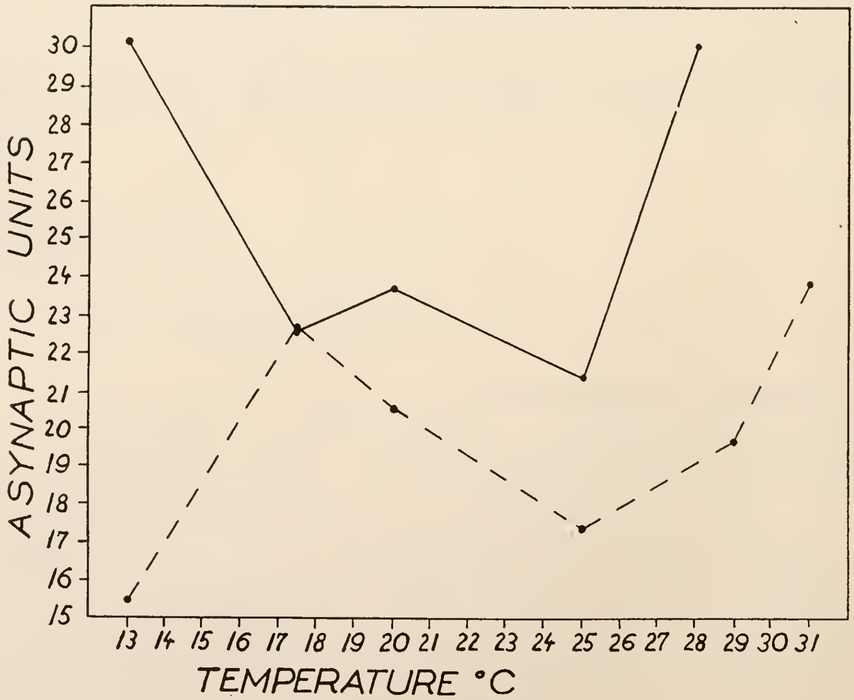

FIGURE 1. Variation with temperature of asynaptic units. Heavy line: Arrowhead × Standard inversion in *D. pseudoobscura*. Broken line: *D. melanogaster* × *simulans* hybrids.

Discussion

Since all the results are based on smears, it might be doubted whether they reflect the true association of homologues in intact nuclei. Hinton (1945) showed that terminal associations are not easily broken, even when stretched with a micromanipulator. This observation makes it unlikely that truly synapsed regions should be separated by the pressure exerted in smearing.

In the two graphs presented in Figure 1, the common features are the minimum of asynapsis at moderate temperatures and the rises on either side of the intermediate range. The most outstanding difference lies in the presence of a second minimum of asynapsis in the *melanogaster* × *simulans* inversion and its absence in the Arrowhead × Standard cross. It has been mentioned that at 13° C., synapsis in the *melanogaster* × *simulans* hybrid is extensive, but not intimate. This phenomenon renders a strict comparison of the two graphs at the 13° C. level impossible.

The presence of a minimum of asynapsis at moderate temperatures in the two rearrangements investigated confirms the findings in Chironomus (Goldschmidt, 1947). However, in view of the considerable differences existing, especially between the *melanogaster* × *simulans* cross and the other two, the combined data do not justify the assumption that we are dealing with a phenomenon universal in structural hybrids. It should be remembered, moreover, that the results obtained with structural hybrids may not have any direct bearing on the process of synapsis in structurally homozygous individuals.

Caution in evaluating the present data seems especially indicated in consideration of the complex evidence regarding the influence of temperature on crossing-over and on chiasma frequency. Plough (1917, 1921) found a minimum of crossing-over at moderate temperatures in the autosomes of *D. melanogaster,* but could not confirm this effect for the X-chromosome in regions distant from the centromere. Stern (1926) found a temperature effect in the X-chromosome near the spindle attachment (from garnet to bobbed). Clark (1943) did not detect any influence of temperature on crossing-over in Habrobracon. White (1934) obtained a curve similar to that of Plough for chiasma frequency in Stenobothrus, but his graphs for Locusta and Schistocerca differ markedly from this pattern. The situation is further complicated by the data of Stern and Rentschler (1936) on somatic crossing-over in the X-chromosome of *D. melanogaster.* This is high at both 17 and 25° C. and low at 30° C.

Much significance has been attributed to the resemblance of Plough's curve to the variation of plasma viscosity with temperature (Heilbrunn, 1928; Frey-Wyssling, 1948). The temperature-viscosity curves of some organisms exhibit several maxima and minima within the biological temperature range. If the absence of a simple linear relationship to temperature may indeed be considered as a common characteristic, this is certainly shared by the graphs presented here. The minimum of crossing-over at intermediate temperatures is paralleled by a minimum of asynapsis in a similar temperature range: a paradoxical situation in view of the fact that chromosomes presumably do not cross-over unless they are previously paired. This paradox might resolve itself if local repulsions between paired homologues proved to be involved in the mechanism of crossing-over.

The influence of temperature on various position effects, previously established by Gowen and Gay (1933, 1934) and various later workers, was studied more recently by Hinton (1949) and Gersh (1949). Their results indicate that with a number of position effects, extreme temperatures produce either smaller or larger effects than moderate temperatures. Since synapsis in some structural hybrids is similarly affected by temperature, it seems possible that some position effects of the variegated type may indeed be connected with synapsis variations, setting up different stresses in the chains of gene proteins.

SUMMARY

1. The effect of temperature on synapsis was studied in salivary glands of *Drosophila pseudoobscura* heterozygous for the Arrowhead inversion, and of *D. melanogaster* × *simulans* hybrids.

2. In the *pseudoobscura* larvae, significantly less asynapsis was observed at 17.5, 20 and 25° C. than at extreme temperatures (13 and 28° C.).

3. In the *melanogaster* × *simulans* hybrids, synapsis was most complete at 13 and at 25° C., and there were two significant peaks of asynapsis at 17.5 and at 31° C.

4. The common feature of the two distributions is the minimum of asynapsis at moderate (20 and 25° C.) temperatures. The possible relationships of this result with the known temperature effects on crossing over and on the position effect are discussed.

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STUDIES ON THE FLAGELLA OF ALGAE. I. GENERAL OBSERVATIONS ON CHLAMYDOMONAS MOEWUSII GERLOFF[1]

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Flagella are the protist prototypes of muscles, presenting perhaps the simplest systems in which we may investigate the processes which knit biochemical structure with the mechanics of motility (*cf.* Dellinger, 1909). The literature on their structure and mode of action in the flagellates has been reviewed by Brown (1945), Owen (1949) and Pitelka (1949). Lowndes (1941) has carried out a series of observations on motility of these organisms, using high-speed cinematography. Tactic behavior patterns in *Chlamydomonas* spp. have been described by Desroche (1912), Kuwada (1916) and Cadoret (1949).

Chlamydomonas moewusii (Chlorophyceae, Volvocales) is a biflagellate unicellular alga which has proved to be a suitable organism for studies of flagellar activity. The presence of chlorophyll enables the cells to multiply in media in which CO_2 is the only source of carbon, and under suitable conditions of illumination their numbers may be quadrupled daily. If their movement is arrested, the flagella may be seen in unstained living cells under high magnifications; they are found not only to be concerned with motility, but also to be involved in a remarkable manner with the processes of mating. Moreover, this species of *Chlamydomonas* is haploid and heterothallic, rendering it suitable for genetic investigation; so that mutants in which motility has suffered some inherent impairment can be crossed and their progeny analyzed for possible recombination, etc.

This paper is to be concerned mainly with general aspects of the flagella of *Chlamydomonas moewusii,* visible under the light microscope in unstained preparations, or in preparations stained with gentian violet following osmic fixation, according to the technique given by Couch (1941). Studies on their fine structure, as revealed by the electron microscope, and on certain physiological aspects of motility and mating are in preparation.

1. Morphology

The two flagella of normal cells arise in the region of a chromophilic body at the anterior extremity of the protoplast, and extend to the exterior through separate perforations of the wall, on either side of the papilla. These pores, in empty walls from which the contents have escaped, are readily visible under the phase-contrast microscope or in electron microscope preparations.

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The flagella themselves are approximately 12–24 μ in length. They vary considerably in older cultures, in which cells with short flagella 2-3 μ in length, and some totally devoid of flagella, may be found on the bottom of the culture vessel. Short flagella may vibrate actively, but the cells are unable to swim. Normal, long flagella are typically geniculate, with a slight bend about 3–4 μ from the point of attachment. Under no conditions has a mastigonema or whiplash been seen, though such a structure has been described for many other species of *Chlamydomonas* (*e.g.*, by Deflandre, 1934; Owen, 1949). In suitably fixed material of *C. moewusii* examined under the electron microscope, the flagella can be seen to terminate in a short mucro, about 0.3–0.4 μ in length.

Preceding vegetative division and shortly before the fusion of gametes, the flagella are withdrawn, the process taking about 30 minutes for completion. In unfavorable conditions, usually leading to death of the cells, the flagella may be shed; almost any irritant (*e.g.*, 10^{-3} *M* acetic acid or 5×10^{-3} *M* ammonia) will produce this effect.

In other conditions, the flagella may undergo a pathological transformation into vesicles flanking the papilla; this transition, which occupies less than a minute, may be observed microscopically. Desroche (1912) found that this change could be induced in *Chlamydomonas obtusa* by subjecting the cells to temperatures close to 40° C. for some minutes. The phenomenon was observed also in *Polytoma* by Francé (1894), Dangeard (1901) and Korschikov (1923), and is discussed by Brown (1945).

Flagella are formed by cells growing on the surface of 1% agar media, and can readily be seen when young cultures are examined *in situ*. On flooding, in darkness or light, the cells are immediately capable of swimming. When the pH of the medium, liquid or agar, falls below 6.0, flagellated cells are replaced during growth by palmella.

2. *Swimming*

Normal flagellar activity has been described for many cells of this type (*e.g.*, Ulehla, 1911). Briefly, it resembles in essentials the movements involved in "breast-stroke" swimming, and is accompanied by a rotation of the whole organism. At 20° C. the rate of flagellar action lies between 10.5 and 12.2 beats per second. (These values were obtained by a simple stroboscopic device in conjunction with dark-field illumination (Richie, 1950) and phase-contrast objectives. Lowndes (1941), using a cinematographic method, observed 8 beats per second in morbid specimens of an unidentified species of *Chlamydomonas*.) Since the position most readily "fixed" by the stroboscope is that in which the flagella extend outward at the sides of the cell (this, too, agrees with the observations of Ulehla, 1911), it is apparent that propulsion is chiefly effected by the downstroke, during which the cell is drawn forward while the ends of the flagella move comparatively little.

Cells of old cultures tend to exhibit a disturbed synchrony of the flagellar pair. One flagellum may cease to beat for a period, or may beat irregularly, with the result that progress is erratic, the cells frequently tumbling and turning in small circles or spinning about the inactive flagellum.

In old cultures one frequently may observe a temporary reversal of the normal direction of progress. For periods of 1–2 seconds, a cell may proceed backwards, without rotating, with its flagella extended parallel from the papilla and vibrating rapidly at the tips. This mode of swimming, characteristic of the related genus *Scourfieldia,* has been observed as sporadic behavior in other species of *Chlamydomonas* (Ulehla, 1911; Oltmanns, 1922).

3. *Creeping*

In examining related organisms (*Polytoma* and *Chlamydomonas* spp.), Francé (1894) and Ulehla (1911) made an interesting observation which has apparently received no attention from later authors. They independently reported that the flagella were capable of creeping along a solid substratum. This has been substantiated by the present author, who first observed it in cells of the paralyzed mutant, M. 236, of *C. moewusii* (Lewin, 1952) in which the flagella do not beat, but extend from the body of the cell. In microscope mounts, the tip of the flagellum can be seen to execute a serpentine motion along the surface of slide or cover-slip; the movement appears autonomous, but ceases as soon as a flagellum becomes detached from its cell. The rate of movement at room temperatures approximates 2 μ per second. If one flagellum is grounded and the other free (or missing), the cell is drawn along thereby; if both flagella extend forward, more or less parallel, the same movement is achieved. However, more usually the flagella are extended in opposite directions, in which case they stretch apart as far as possible, while the cell remains stationary, with its papilla pressed against the substratum. This accounts for the frequency with which one encounters *Chlamydomonas* cells end-on in microscopical preparations, as figured, for example by Gerloff (1940, Figs. 1 and 2).

Two possible mechanisms might be conceived to account for this movement:

1. The action of secondary cilia might be postulated.
2. Peristaltic waves or undulations may be propagated basipetally from the tips.

The first possibility is in this case immediately excluded since the surface of the flagella is seen under electron microscopy to be quite devoid of lateral appendages. This is in agreement with all other observations on the flagella of members of the Volvocales (*cf.* Brown, 1945).

The possibility then remains that the creeping of the flagella is attributable to pulses travelling down from the tip—which unfortunately has not been visibly confirmed, since the flagella are considerably less than 1 μ in width. In fact, Ulehla (1911) described in non-beating flagella of *Gonium* the uninterrupted passage of two or more spiral waves within a flagellar sheath. Their movement, however, was acropetal.

4. *Mutants*

Mutants in which the flagella are lacking altogether (though the basal chromophilic body remains), and others in which the swimmming (but not the creeping) faculty is lost, have been obtained following ultra-violet irradiation and are described elsewhere (Lewin, 1952).

5. *Mating behaviour*

a. Clumping. The species is regularly heterothallic and isogamous: complementary mating types have been arbitrarily designated as "plus" and "minus." When suspensions containing sexually active plus and minus cells are mixed, the phenomenon of clumping occurs. It can be observed that in this species only the smaller, younger cells take part in this sexual response, while the larger cells are capable only of vegetative reproduction. The young cells may be said to be facultatively gametic, and it is only when in this state that they are capable of aggregating into clumps. Clumping is attributable to a specific "stickiness" of the gamete flagella, by which cells of opposite mating-type become attached to one another (Gerloff, 1940). The aggregation appears to follow random contacts; in dilute suspensions the cells tend to associate in twos and threes, while in crowded conditions clumps of 50 or more cells may form within 1–2 minutes of mixing, and may continue to grow for several minutes.

b. Pairing. The specific stickiness of the flagella of gametes during pairing can be compared, perhaps, to an antigen-antibody association between proteins, resulting initially in adherence of the flagella at any point of contact. Furthermore, a *polar* orientation of the pairing flagella is induced, so that they come to lie parallel with one another while extending in the same direction relative to their respective cells. In other words, they orient distal to distal end, proximal to proximal.

By some unascertained means, cells within a clump succeed in coupling so that both flagella of the partners are paired; probably this takes place during the random flailing of these organelles. They do not become entangled (as has been stated in the literature for other species—probably incorrectly), but adhere for a large part of their length. According to the position of the original points of adherence, the relation of the pairing cells will be at first more or less skew (*i.e.,* not coaxial), and now a further organizing mechanism comes into play. As has already been mentioned, the individual flagella have an ability to creep over surfaces. This faculty now enables pairing cells to adjust their spatial relations, by sliding their flagella along those of the partner, until their respective papillae are in apposition. Such sliding adjustment may perhaps expain the polar association of flagella, mentioned above. Flagella may possibly pair at first in an antipolar (*i.e.,* distal-to-proximal) fashion, but disengage shortly afterwards at their free ends. This, however, has not been observed.

The cells become appressed, papilla to papilla, and about 5 minutes after the initiation of mating a protoplasmic bridge is formed between their anterior ends (*cf.* Korschikov, 1927). Another 5 minutes later, the flagella suddenly lose their stickiness and, with it, any tendency for the pair to remain with others in a clump. The four flagella of the copulants disengage to extend freely in the medium, and the pair soon swims away.

c. Behavior of pairs. During the changes related in the previous section, the cells have been engaged in much to-and-fro movement within a struggling mass, and this apparently disorganized activity persists for a minute or two in newly-united pairs. Its place is very soon taken, however, by a regular, uni-directional mode of swimming, in which the pair behaves as a single entity and the conflicting activity of the two sets of flagella is no longer, or only sporadically, apparent. In some way, therefore, the motor apparatus of the two copulants has become adjusted so that directed locomotion (*e.g.,* positive phototaxis) is once more feasible. The natural advantage of such a coordinating device is clear.

Close examination of such pairs reveals two notable features: (1) Whenever a slight discrepancy in size, form or color enables one to distinguish the partners one from the other, it can be observed that the relationship of the two cells is constant. One cell retains its anterior position, while its mate remains in the rear, throughout the swimming period of the pair. (2) The flagella of the posterior partner are wholly responsible for the motility of the pair, beating in apparently normal fashion. Those of the anterior cell, their plane now perpendicular to that of the driving cell (as observed in *Phyllocardium* by Korschikov, 1927), extend along the body of the partner, and rarely exhibit more than a slight twitching of the tips.

To establish whether the differentiation in flagellar activity is genetically inherent in the respective mating-types, the cells were labelled in various ways, so that they could be distinguished by visible microscopic characters. Lerche showed how this could be done in *Dunaliella,* by mixing starved gametes of one mating-type, in which a red carotinoid had accumulated, with well-nourished green cells of the opposite type (Lerche, 1937, Plate 6).

In *C. moewusii* it has been possible to carry out similar observations, by mating starved cells with normal cells. Alternatively, the gametes have been rendered distinguishable by intravital staining (*e.g.*, with dilute neutral red), or by employing genetic markers. Strains in which the cells are spherical instead of oblong, in which abnormal quantities of intracellular volutin are accumulated, or in which the flagella are paralyzed, have all been used in such experiments (Lewin, 1950). Observations on reciprocal combinations between suspensions of gametes labelled in these various ways have shown that it is the plus partner which, in the posterior position, is responsible for the propulsion of the pair. The minus partner, shortly after the formation of the intercellular bridge, ceases its flagellar activity (except for rare erratic bursts in the early stages of pairing). This difference in behavior is not dependent on the physiological state of the cells, but is genetically inherent in the mating types.

SUMMARY

1. Cells of *Chlamydomonas moewusii* normally swim by a synchronized backward beat of the paired flagella.

2. When appressed to solid substrata, the flagella are capable of an independent creeping movement. This faculty does not persist in detached flagella.

3. During mating, cell clumps are formed by the adhesion of flagella of cells of opposite mating-types.

4. By means of the flagella, copulating cells can adjust their relative positions until cytogamy can be initiated.

5. Paired cells do not fuse for some hours, during which swimming results from the activity of one partner only.

6. By suitable labelling, it can be shown that the flagella of the plus partner remain active, while those of the minus cell cease to beat after pairing.

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TEMPERATURE REQUIREMENTS FOR MATURATION OF GONADS OF NORTHERN OYSTERS

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Gonad development and spawning of oysters (*Crassostrea virginica*) of our North Atlantic coast are well-defined seasonal phenomena. For example, in Long Island Sound, where extensive oyster beds are located, spring gonad development commences during the first part of May and spawning begins during the end of June or the first week in July (Loosanoff and Engle, 1942). There is no doubt that the rate of progress of gametogenic activities and the beginning of spawning of oysters are to a large extent influenced by the temperature of the surrounding water.

In the opinion of many students the period required for maturation of the gametes of the European oyster, *O. edulis*, is a function of time and temperature (Voisin, 1933; Orton, 1937; Korringa, 1941, and others). Few studies of a similar nature have been made, however, on the American oyster, *C. virginica*. Perhaps the only works dicussing effects of temperature on gonad development were those of Nelson (1928a), who thought that spawning of *C. virginica* should be expected approximately 160 degree-hours after the temperature of the water reaches and remains at the 20.0° C. level, and of Loosanoff (1942), who described seasonal gonadal changes of oysters of Long Island Sound and referred to the temperatures of the water prevailing during the different stages of gonad development and at spawning. In general, however, many aspects of the effect of temperature on the gonad development of *C. virginica* remained unknown.

The experiments described in this article were designed to ascertain the number of days needed for oysters of both sexes kept at different but constant temperatures to develop first mature cells; to reach the physiological state at which they can be induced to spawn by thermal or chemical means; and to reach the condition at which they will be ready to spawn normally, without any artificial stimulation. We hope that our article will contribute toward the understanding of the dependence of individuals and of populations upon ecological factors, such as temperature.

We wish to express our appreciation to our colleagues, Charles A. Nomejko, for assisting us in different phases of these studies, to David W. Calhoun for the statistical treatment of the data, and to Professor Thurlow C. Nelson of Rutgers University for reviewing the manuscript.

METHOD

The oysters used in these experiments ranged in age from three to five years. They were collected in winter when the temperature of the water over the beds was near 0.0° C. In the laboratory the oysters were separated into five groups and placed in trays with running, cold sea water having a temperature of about 8.0° C., just high enough to allow the oysters to come out of hibernation. Twenty-four hours later the temperature of the water running in the trays was raised within 4 to 8 hours to 10.0, 15.0, 20.0, 25.0 or 30.0° C. and steadily maintained, within ± 1.0°C., at those levels until the end of the experiment.

In the first series of experiments small samples of oysters consisting of 5 to 15 specimens were taken at 5-day intervals for gross examination and histological studies (Loosanoff and Davis, 1949). In later experiments, however, each sample consisted of 50 oysters and when needed the oysters were examined daily. The quantitative data of this article are based on the latter samples.

The method of conditioning oysters to develop spawn in winter was, in principle, the same as that described several years ago (Loosanoff, 1945). It was modified only in the respect that instead of keeping the oysters in aquaria, the water of which was changed at certain intervals, they were kept in shallow trays through which water of the desired temperature was running continuously. The oysters were fed a rich culture of plankton which was automatically and continuously added to the running water.

Examination of the oysters was made according to the following procedure: Before the oysters were ripe enough to be induced to spawn, all 50 individuals constituting each sample were opened and the content of their gonads examined under a microscope to determine the number of males with active sperm, number of females with fertilizable eggs, and the number of immature oysters (Tables I–IV). All males containing active sperm were considered physiologically mature although not necessarily ready to spawn. Possession of fertilizable eggs was determined by adding mature sperm to the egg suspension made from each female containing large ovocytes. Formation of the polar body was taken as the criterion that the eggs were fertilizable.

After the oysters were kept long enough at the conditioning temperatures to be approaching the state in which they could be induced to spawn the procedure of the examination was changed as follows: Each of the 50 oysters constituting a sample was placed in a separate spawning dish, which was filled with water of the same temperature as that at which that group of oysters was conditioned. Then all the oysters were subjected to chemical stimulation consisting of the addition of sperm and egg suspensions to the water in the spawning dishes.

Ninety minutes after the beginning of the chemical stimulation all the oysters that failed to respond were further stimulated by an increase of the water temperature. After another period of 90 minutes the oysters that failed to spawn were opened and their gonads examined for the presence of active sperm or fertilizable eggs.

OBSERVATIONS

Control samples

Gonads examined at the beginning of the experiment contained small undeveloped follicles typical for hibernating oysters (Loosanoff, 1942). The male sex cells were in the early stages of spermatogenesis, predominantly spermatogonia or early spermatocytes. In the females the follicles contained mostly indifferent cells, some ovogonia and a few young ovocytes 15–18 μ in diameter. In both sexes most of the space between the body wall and the digestive diverticula was occupied by the vesicular connective tissue which surrounded the small islands formed by the follicles (Fig. 1). The oysters were in good condition and contained large quantities of glycogen.

10.0° C. group

Even the most advanced oysters of this group, examined 35 days after the beginning of the experiment, showed only a slight development of their gonads. In the females the largest ovocytes were only 18–20 μ in diameter and contained virtually no yolk (Fig. 2). In the males, there were secondary spermatocytes and a few spermatids, but no spermatozoa. Even in these individuals, which constituted about 5 to 6 per cent of the samples, such development took place only towards the end of the experiment. In the other oysters of this temperature group the gonads even then resembled the winter condition.

It is interesting that in the 10.0° C. group the anastomosis of the follicles and gametogenesis were so slow even though these oysters, judging by the quantities of feces produced, were feeding more actively than those kept at the temperature of 25.0 or 30.0° C. Moreover, at the end of the experiment the meats of the low-temperature oysters still contained approximately as much glycogen as they had at the beginning. Considering these conditions, which indicated that the oysters were kept in a relatively favorable environment, it seems apparent that the depressed gonad development of those oysters was principally due to the low temperature.

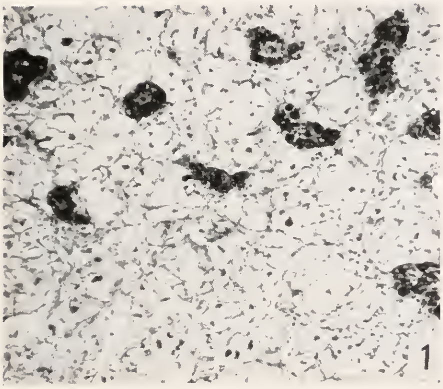
TABLE I

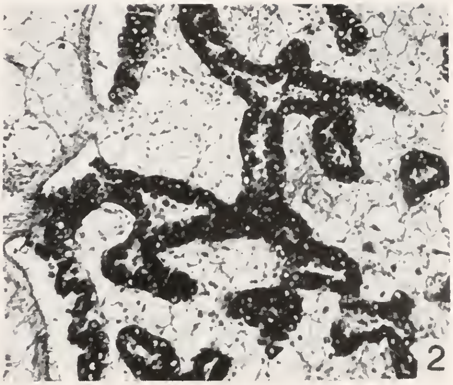
Number and per cent of oysters, kept for different periods at 15.0° C., that were induced to spawn, or showed presence of mature gametes. Number and per cent of immature oysters and those with sex undetermined are also given. Each sample composed of 50 oysters.

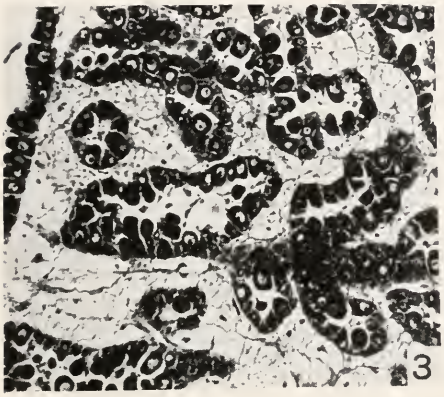
Days of conditioning	Induced to spawn				Unspawned				Per cent		
	By chemical stimulation		By chemical and thermal stimulation		Mature gametes		Immature gametes		Spawned	Unspawned but with mature gametes	Immature
	Male	Female	Male	Female	Active sperm	Fertilizable eggs	Sex recognizable but immature	Sex undetermined			
10	—	—	—	—	5	0	11	34	—	10	90
15	—	—	—	—	6	0	14	30	—	12	88
20	—	—	—	—	6	3	16	25	—	18	82
25	—	—	—	—	16	6	10	18	—	44	56
30	0	0	0	0	26	9	11	4	0	70	30
35	0	0	2	1	17	17	7	6	6	68	26
40	0	0	3	0	19	19	7	2	6	76	18
45	0	0	4	2	16	20	7	1	12	72	16
55	1	0	14	5	3	27	0	0	40	60	0

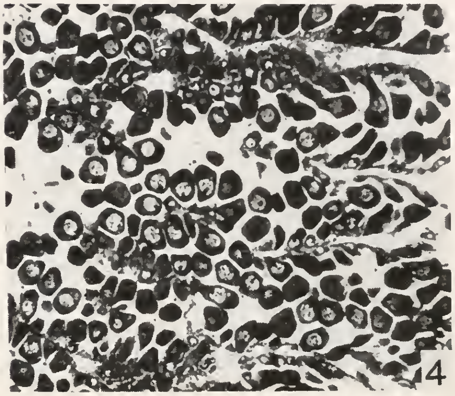
15.0° C. group

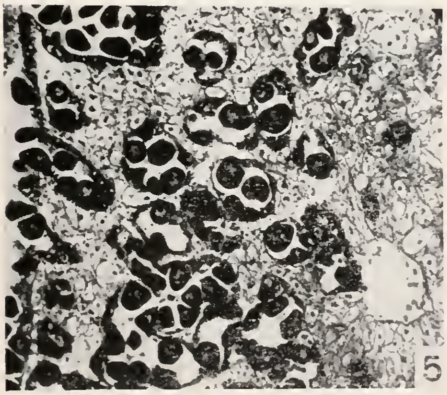
During the first five days of exposure the oysters showed little gametogenic activity and their gonads still remained in winter condition. The advanced males











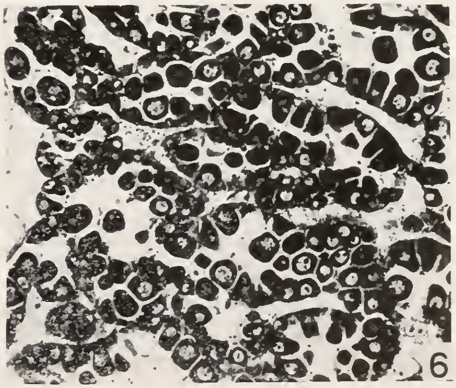


FIGURE 1. Winter gonad of oyster showing undeveloped follicles surrounded by connective tissue. × 112.

FIGURE 2. Gonad of most advanced female oyster of the group kept 35 days at 10.0° C. × 112.

FIGURE 3. Gonad of female oyster showing proliferating follicles containing growing ovocytes; 15 days at 15.0° C. × 112.

FIGURE 4. Gonad of female oysters; 40 days at 15.0° C. × 112.

FIGURE 5. Partly discharged gonad of female oyster; 55 days at 15.0° C. × 112.

FIGURE 6. Gonad of female oyster approaching ripeness; 13 days at 20.0° C. × 112.

showed a slight proliferation of follicles but gametogenesis proceeded only as far as the formation of spermatids. After 10 days the female oysters still showed little progress, while in the males a slight anastomosis of the follicles was noticed and in five of them a few spermatozoa were found (Table I). Towards the 15th day the largest ovocytes were already about 35 μ in diameter, but the vesicular connective tissue still occupied a large part of the interfollicular spaces (Fig. 3).

By the 20th day 9 oysters, representing 18 per cent of the sample, contained active sperm or fertilizable eggs, while 16 other oysters possessed recognizable sex cells (Table I). Thus, on this date the sex of 50 per cent of the oysters composing the sample was already recognizable.

On the 25th and 30th days attempts were made to induce the oysters to spawn. They were first stimulated by the addition to the water of a suspension of ripe spermatozoa or eggs, and later by a rapid increase of the temperature to about 32.0° C. Some oysters, probably females, moved their shells in typical rhythmic spawning motions, but no eggs were discharged.

Spawning was successfully induced for the first time on the 35th day (Table I). However, of 50 oysters composing the sample only three, two males and one female, spawned and then only after the combined effects of chemical and thermal stimulations. The eggs obtained in this spawning developed into normal larvae.

By this time a difference in the gonad development of the individuals composing the samples became especially pronounced. The thickness of the gonadal layer varied from almost 0.0 to 3.0 mm. and while the most advanced oysters could be induced to spawn, the least advanced possessed gonads so undeveloped that their sex could not be easily determined (Table I).

Between the 35th and 45th days the oysters continued to develop, their enlarged gonadal follicles coming in contact with each other, while the vesicular connective tissue, which was so prominent earlier in the development, had largely disappeared (Figure 4). Yet, even on the 45th day none of the oysters could be induced to spawn by chemical stimulation alone.

After 55 days all the oysters had either fertilizable eggs or active sperm, and 20 of them, constituting 40 per cent of the sample, were induced to spawn (Table I). Of these, however, only one responded to chemical stimulation alone, while all others required the rapid increase in temperature. It is possible that this happened because several oysters in the sample had already discharged some spawn, and were, therefore, comparatively indifferent to the stimulating effect of sex products. That some of the oysters may have spawned was shown by histological examination of the gonad tissue taken from the oysters of an auxiliary experimental group, some of which showed partially discharged gonads, characterized by contracting follicles and the invasion of connective cells into the interfollicular spaces (Fig. 5).

At the end of the 90-day period an egg and sperm suspension was added to the trays to determine if the oysters would respond to this stimulation and begin spawning, even if there were no increase in temperature. Within a few minutes after the addition of the suspension almost all the oysters in the tray commenced spawning and continued to do so for about two hours. During the spawning the temperature steadily remained at 15.8° C. The eggs collected from this spawning developed into normal larvae.

20.0° C. group

The stimulating effect of this temperature was clearly seen early in the experiment. At the end of the first five days 28 per cent of the oysters already contained either active sperm or fertilizable eggs (Table II). After eight days the oysters were first induced to spawn by a combination of chemical and thermal stimuli, and two days later some oysters responded to chemical stimulation alone (Table II). Although some ripe eggs were found in the females after only five to ten days of exposure, nevertheless, such eggs still represented a minority of the follicular cells.

Table II

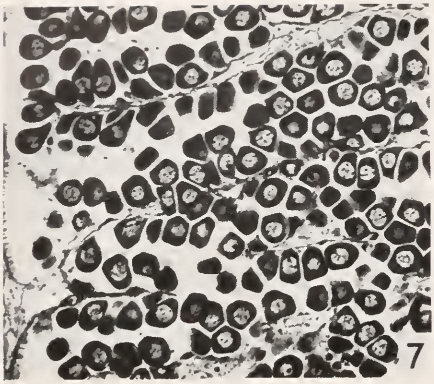
Number and per cent of oysters, kept for different periods at 20.0° C., that were induced to spawn, or showed presence of mature gametes. Number and per cent of immature oysters and those with sex undetermined are also given. Each sample composed of 50 oysters.

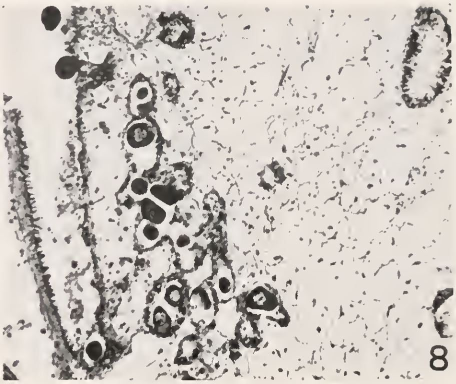
Days of conditioning	Induced to spawn				Unspawned				Per cent		
	By chemical stimulation		By chemical and thermal stimulation		Mature gametes		Immature gametes		Spawned	Unspawned but with mature gametes	Immature
	Male	Female	Male	Female	Active sperm	Fertilizable eggs	Sex recognizable but immature	Sex undetermined			
5	0	0	0	0	9	5	7	29	0	28	72
8	0	0	2	0	14	4	18	12	4	36	60
10	2	0	11	0	9	7	14	7	26	32	42
13	1	0	7	8	8	16	9	1	32	48	20
15	3	3	9	6	4	14	10	1	42	36	22
18	6	6	3	11	8	12	2	2	52	40	8
20	1	1	16	12	1	18	1	0	60	38	2
25	0	0	19	6	4	21	0	0	50	50	0

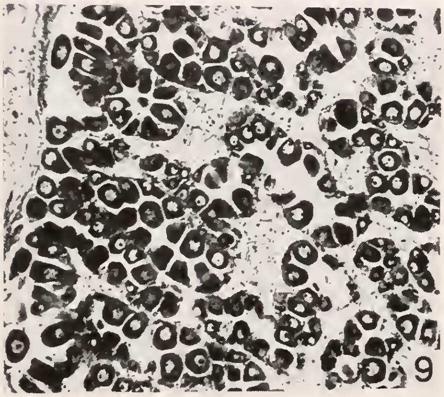
Again, as in the preceding temperature classes, the individual differences among the oysters of the same group were striking. While 26 per cent of the oysters kept at 20.0° C. for ten days could be induced to spawn, 7 others still possessed gonads resembling the winter condition (Table II). This condition of slow gonad development persisted even though the oysters were feeding vigorously and showed rapid growth of shell. Similar individual differences in gonad development were also found in oysters growing under natural conditions in Long Island Sound (Loosanoff, 1942).

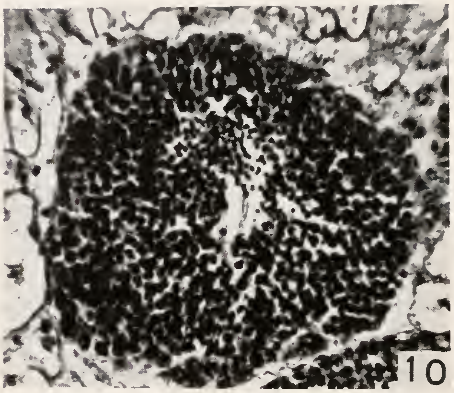
The spawning behavior of the females within the samples also showed that they were in physiologically-different states of ripeness. Some of them did not respond at all to stimulation. These were usually immature individuals, the sex of which could not be determined. Others moved their shells as in spawning but released no eggs. Still others discharged a few apparently immature eggs measuring only 45 μ, *i.e.*, about 5 μ smaller than normal eggs. Finally, there were females that discharged normal eggs which developed into healthy larvae. The ratio between these groups changed continuously, as can be judged from the data in Table II.

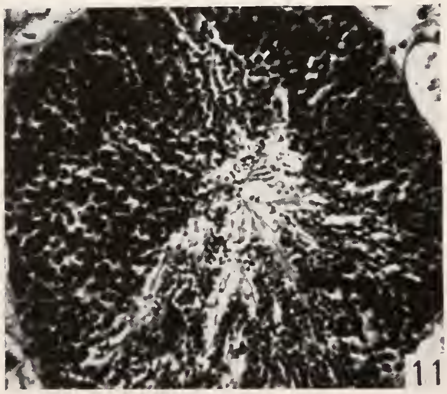
Between the 13th and 18th days many oysters were either approaching ripeness (Fig. 6) or were ripe (Fig. 7). At the latter date 52 per cent of the oysters











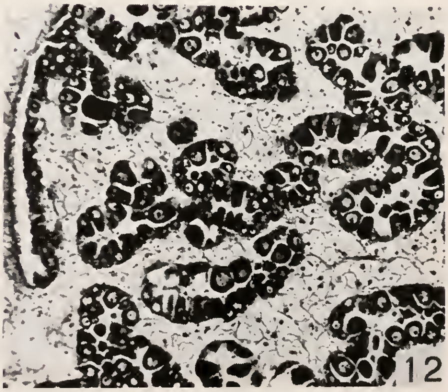


FIGURE 7. Gonad of ripe female oyster; 18 days at 20.0° C. × 112.

FIGURE 8. Gonad of female oyster in advanced stage of spawning; 25 days at 20.0° C. × 112.

FIGURE 9. Gonad of female oyster containing some ripe eggs but still not quite mature; 8 to 10 days at 25.0° C. × 112.

FIGURE 10. Gonadal follicle of male oyster showing few ripe spermatozoa in center of lumen; 3 days at 30.0° C. × 475.

FIGURE 11. Gonadal follicle of male oyster; 5 days at 30.0° C. × 475.

FIGURE 12. Gonad of female oyster containing some fertilizable eggs; 5 days at 30.0° C. × 112.

spawned, when stimulated, and the number of individuals with sex undetermined was only one or two per sample of 50. After 20 days 98 per cent either spawned or contained ripe gametes.

On the 20th day of exposure the number of oysters that could be induced to spawn showed a considerable decrease, as compared with the record of the 18th day (Table II). Between these two dates some of the oysters had apparently spawned in the natural, unprovoked way, and the presence of their sex products in the surrounding water may have made them unresponsive to this type of stimulation when it was applied experimentally a day or so later. This contention is supported by the observation that on the 25th day of the experiment none of the oysters could be induced to spawn by chemical stimulation alone. Histological studies of their gonads also showed that some were already in the typical post-spawning condition characterized by contracted follicles still retaining some ripe eggs, a large number of phagocytes, and a rapid invasion of connective tissue throughout the space between the body wall and the digestive diverticula (Fig. 8). Such oysters, obviously, could not be easily stimulated and, therefore, were not included in the number of oysters induced to spawn on that date. Instead, they were classified together with those that were unspawned but contained active sperm or fertilizable eggs. This explains why on the 25th day of exposure the number of oysters that could be induced to spawn showed a decrease, while the number of individuals which were unspawned but contained ripe gametes showed an increase (Table II).

25.0° C. group

In this group rapid gametogenesis and proliferation of gonadal follicles began within the first few days. After five days 17 oysters, constituting 34 per cent of the sample, already contained mature gametes (Table III). Of these 13 were males containing active sperm and the rest were females with fertilizable eggs. In both sexes, however, there were only a few ripe cells, indicating that the oysters were just entering the state of ripeness.

TABLE III

Number and per cent of oysters, kept for different periods at 25.0° C., that were induced to spawn, or showed presence of mature gametes. Number and per cent of immature oysters and those with sex undetermined are also given. Each sample composed of 50 oysters.

Days of conditioning	Induced to spawn				Unspawned				Per cent		
	By chemical stimulation		By chemical and thermal stimulation		Mature gametes		Immature gametes		Spawned	Unspawned but with mature gametes	Immature
	Male	Female	Male	Female	Active sperm	Fertilizable eggs	Sex recognizable but immature	Sex undetermined			
5	—	—	—	—	13	4	10	23	—	34	66
6	0	0	0	0	19	8	11	12	—	54	46
7	0	0	8	4	7	13	10	8	24	40	36
8	5	1	3	1	13	17	10	0	20	60	20
9	1	3	7	5	8	13	9	4	32	42	26
10	9	13	3	5	5	10	3	2	60	30	10

By the end of the seventh day some of the oysters were induced to spawn by the combined effects of chemical and thermal stimuli, and the next day five males and one female responded to chemical stimulation alone.

After 9 and 10 days of conditioning the samples gave 32 and 60 per cent of spawners respectively, and at the latter date the number of immature oysters was only 10 per cent (Table III). However, some of those that were capable of spawning still possessed gonads containing many immature cells. This condition is well illustrated in Figure 9, where together with mature ovocytes there are many unripe cells. Furthermore, the large quantity of connective tissue in the interfollicular spaces indicated that further growth of the follicles was still to occur.

Mass spawning of the oysters of this temperature group took place during the eleventh day. In some earlier experiments, however, mass spawning of one group kept at 25.0° C. occurred on the ninth day (Loosanoff and Davis, 1949). In both cases the spawning was unprovoked since the temperature remained steadily at 25.0° C. and no sex products capable of stimulating the experimental oysters could be present in the water flowing into the trays, because the experiments were conducted in February when the oysters in the Harbor, from which our water supply is obtained, were hibernating and, obviously, could not release any spawn.

30.0° C. group

This group, as a whole, responded extremely rapidly to the effect of high temperature and after three days of exposure 18 per cent of the oysters already possessed physiologically-ripe cells (Table IV). Although the most advanced females showed expanding follicles already containing a few fertilizable eggs, their gonads, nevertheless, still had a large quantity of connective tissue separating the follicles. In the males rapid spermatogenesis was in progress, already resulting in a few ripe spermatozoa in the centers of the lumens of the follicles (Fig. 10).

TABLE IV

Number and per cent of oysters, kept for different periods at 30.0° C., that were induced to spawn, or showed presence of mature gametes. Number and per cent of immature oysters and those with sex undetermined are also given. Each sample composed of 50 oysters.

Days of conditioning	Induced to spawn				Unspawned				Per cent		
	By chemical stimulation		By chemical and thermal stimulation		Mature gametes		Immature gametes		Spawned	Unspawned but with mature gametes	Immature
	Male	Female	Male	Female	Active sperm	Fertilizable eggs	Sex recognizable but immature	Sex undetermined			
3	—	—	Not Employed		7	2	3	38	—	18	82
4	—	—			26	3	6	15	—	58	42
5	16	3			7	3	8	13	38	20	42
6	15	12			10	1	8	4	54	22	24
7	22	13			1	2	6	6	70	6	24

After five days the oysters were successfully induced to spawn. Because they were conditioned at the temperature of 30.0° C. it was impractical to use a much higher temperature to induce spawning and, therefore, chemical stimulation alone was used. Nineteen oysters of the total number of 50 spawned but the quantities of spawn released were still small. Histological examination of the gonads showed that although the males conditioned at 30.0° C. for five days (Fig. 11) had considerably more mature sperm than after three days (Fig. 10), the unripe cells, nevertheless, still predominated. The same could be said for the females in which ripe ovocytes were not too common (Fig. 12).

After six days 54 per cent of the oysters were induced to spawn. Yet, 24 per cent were still immature and in four per cent the sex could not be determined (Table IV). After seven days 70 per cent could be spawned, and in one of the experiments an unprovoked mass spawning took place at the end of the seventh day. Both sexes participated in the spawning, the males discharging large quantities of sperm, but the number of eggs released still remained comparatively small.

By the tenth day the gonads of the oysters were already in advanced post-spawning stages, characterized by contracted follicles similar to those shown in Figures 5 and 8.

Discussion

As has already been mentioned, in studying the maturation of gonads of oysters kept at different but constant temperatures we were primarily interested in the number of days needed for formation of the first physiologically-mature gametes in each sex. We were also interested in finding how soon oysters can be induced to spawn by artificial means, *i.e.*, by the addition of sex products or by a rapid increase in temperature and, finally, how soon they would begin to spawn normally, without the help of any artificial stimulation.

Our experiments showed that the temperature of 10.0° C. is not high enough to induce gametogenic activities in most of the oysters, and in those few in which such activities were initiated, the development proceeded at an extremely slow rate. Apparently this temperature was too low to permit the conversion of glycogen and other materials stored in the oyster bodies into sex products. While the exact mechanism of this complex process is still not understood, it is thought that it involves the action of a certain enzyme or chain of enzymes, which is retarded, or completely inhibited, at low temperatures.

There are ample indications that some processes in animals, including oysters, are slowed down by low temperature more than others, thus showing that the effect of such low temperature is, obviously, differential. For example, while the growth of the shell of the oysters of our waters may proceed even at a temperature of one or two degrees below 10.0° C., the gonad development at such a temperature is either entirely arrested or proceeds at such a slow rate that a successful completion of that process cannot be expected. Perhaps this is a good example of the so-called developmental or biological zero of Bělehrádek (1935) who considers such a zero as the highest temperature threshold at which a certain protoplasmic activity is still arrested by cold.

Our observations, that at a temperature of 10.0° C. even the most advanced oysters did not form spermatozoa after 35 days of exposure, differ from those of Nelson (1928b) who, in his studies of oysters in Barnegat Bay, found that "active spermatozoa may be found in a few oysters at temperatures as low as 9°–10° C., Table I." Nelson's table shows, however, that the temperature data which he offers were the averages for the week preceding the date of collection of the gonad samples. It is possible, therefore, that the maximum temperature during, or prior to, this period exceeded 10.0° C., causing spermatogenic activities which resulted in the formation of a few spermatozoa. Nevertheless, the results of our controlled experiments are in full agreement with the field observations of Nelson (1928a) who emphasized the wide individual differences often found in the condition of the gonads of oysters collected at the same time and from the same bed.

The experiments also demonstrated that under certain conditions ripening of gonads and spawning of oysters of both sexes may be accomplished at a temperature of only about 15.0° C. These experiments prove rather conclusively that the old conception that 20.0° C. is the minimum temperature at which spawning of *C. virginica* is possible does not hold for the oysters of Long Island Sound.

Laboratory observations on ripening of gonads at such a relatively low temperature corroborate the studies in Long Island Sound, which showed that spawning of oysters may take place at a temperature as low as 16.4° C. (Loosanoff, 1939). Recently we have found that in nature oysters may possess ripe gonads in early summer when the temperature of the surrounding water is only 14.6 to 15.4° C. On July 1, 1948 samples of oysters were taken from two of our collecting stations located in Long Island Sound at a depth of 30 feet. At the time of collection the temperature of the water at the first station was 14.3° and at the second, 15.4° C. The highest temperature recorded that summer at the first station, prior to collection of the sample, was 14.6° C., while at the second station it was 15.4° C. These oysters were placed in the laboratory in separate trays of slowly running water at about 22.0° C. A few hours later the oysters began to spawn copiously, both sexes participating in the act. The fertilized eggs were cultured and became normal larvae.

A similar experiment was repeated in early June, 1949. The oysters were collected from a depth of 35 feet, where the highest temperature recorded for the season was only 14.5° C. Again the oysters spawned a few hours after they were placed in warm water. Thus, the oysters were near the spawning condition at the time of collection even though the highest temperature the water had reached that season was only about 15.0° C.

Since spawning is possible at temperatures lower than 20.0° C., Nelson's (1921) conclusion that if the temperature of the water fails to reach 70.0° F., (21.2° C.) and remain at that level for some time, the oysters will not spawn at all, should be regarded as not applicable to the oysters of Long Island Sound. For the same reason Nelson's (1928a) suggestion that spawning of *C. virginica* may be expected to begin approximately 160 degree-hours after the temperature of the water has reached 20.0° C. is not true for Long Island Sound oysters. We think that the discrepancies between our observations and Nelson's may be explained on the basis that the two studies were made in geographical areas where ecological conditions are different, and where the local populations of oysters may possess significantly different physiological traits. That such physiologically-different groups of oysters may exist was recently shown by Stauber (1950) who by reviewing published and some unpublished records concluded that there appear to be three physiological races of oysters, *C. virginica*, which require different minimum temperatures at which they are capable of spawning. Stauber showed that the race with the lowest critical temperature is not found in Canada, at the northern limit of the geographical range of the species, but in Long Island Sound, some 600 miles airline farther south. Loosanoff and Nomejko (1951), by conducting experiments in Milford Harbor, Connecticut, with oysters brought from different geographical areas along the Atlantic coast, found that the breeding temperature requirements of the northern oysters were somewhat lower than those of the southern groups, thus corroborating Stauber's conclusions and suggesting once more that some physiological requirements of oysters of different geographical districts may be decidedly different. We would also like to mention that our colleague, Professor Thurlow C. Nelson, called our attention to the most interesting fact that many years ago Lamarck from shell characteristics alone designated the Long Island Sound oyster as *Ostrea borealis,* which he believed to be a species distinctly different from *O. virginica* (now *Crassostrea virginica*).

TABLE V

Per cent of oysters of both sexes with mature gametes and estimated number of females in samples of 50 oysters kept at temperature of 15.0, 20.0, 25.0 or 30.0° C. for different numbers of days. Calculated days' exposure compensated for inequalities in sex ratio are also shown.

Days exposed	Temperatures in °C.											
	15.0			20.0			25.0			30.0		
	Compensated days exposed	% Oysters with mature gametes	Estimated number of females	Compensated days exposed	% Oysters with mature gametes	Estimated number of females	Compensated days exposed	% Oysters with mature gametes	Estimated number of females	Compensated days exposed	% Oysters with mature gametes	Estimated number of females
3										3.0	18	27
4										5.2	58	18
5				4.9	28	28	4.9	34	28	5.7	58	23
6							6.1	52	27	6.7	76	24
7							6.1	64	33	7.6	76	25
8				7.8	40	29	7.8	80	29			
9							7.9	74	33			
10	9.8	10	29	10.6	58	26	8.8	90	33			
13				11.2	80	34						
15	14.5	12	30	12.9	78	34						
18				16.1	92	33						
20	17.4	18	34	18.4	98	32						
25	25.9	44	28	26.6	100	27						
30	35.9	70	23									
35	35.6	74	29									
40	43.2	82	27									
45	44.9	84	30									
55	52.3	100	32									

As is well known, the rate of metabolic processes of an animal increases almost to the extreme upper limit of the temperature at which this animal carries on its activities. This is especially well noticed in poikilothermous animals, the body temperature of which is usually at or near that of their environment. To express more or less accurately the influence of temperature on biological phenomena and to demonstrate the relation existing between temperature and the speed of physiological processes, a number of formulae have been proposed by many students including the most often quoted Krogh (1914), Arrhenius (1915) and Bēlehrádek (1935). It is not the purpose of our article to evaluate the relative merits of the different formulae, except to mention that although there is not a universally acceped mathematical expression for the effect of temperature upon the rate of biological processes, it is widely recognized, nevertheless, that some of the formulae, including those mentioned above, offer biologists a tool for an approximate solution of the problems and, at times, for prediction of the results of the studies concerning relationships between temperature and physiological processes, such as gonad development or spawning.

Choice of indices of the gonadal development of a group of oysters is somewhat arbitrary. The two most satisfactory for examination were the number of individuals with active sperm and the number with fertilizable eggs. Accordingly, an analysis was made of the sums of these two indices, as a percentage in each sample of 50 oysters (Table V). This percentage is hereafter called the degree of maturity of the sample. It was then transformed to the probit scale, and compared with the exposure time on a logarithmic scale. The four groups of samples at the different temperatures gave four series of points. By the method of maximum likelihood four parallel straight lines with the slope 4.343 were fitted, one to each series. Deviations did not appear to come from systematic curvature or non-parallelness of the fitted lines.

This analysis indicated that (1) since straight lines follow use of the log-time scale, individual differences may be explained by variability in the rates of development of different individuals. (2) Parallelism of the four lines is evidence that a given temperature increase affects all individuals in the same way, accelerating their rates of development by the same factor. (3) The slope of the fitted lines measures the variation of the oyster population with respect to differences in time required to reach maturity. For example, at a given temperature, it will take the slowest developing 20 per cent of the population over 2.5 times as long to reach maturity as it does the fastest 20 per cent (Fig. 13).

Our experiments have shown that males mature earlier than females (Tables I–V). Therefore, we would expect that samples largely female would be slow and those predominantly male would be farther advanced than the average. Since the sex of oysters with poorly developed gonads could not be recognized, the actual number of each sex in some samples was unknown. Hence, the sexes could not be analyzed separately, and random fluctuations in sex ratio have presumably disturbed the temperature averages, making comparison uncertain. A refinement of the analysis was obtained by allowing for the estimated proportion of females in each sample. An appropriate allowance was then subtracted from the actual time for predominantly female samples and added to the time of the samples with more than the average number of males.

Estimated sex ratios in each sample were based on the preliminary fitted lines, by dividing the number of sexually indeterminate individuals roughly in proportion to the expected unripe fractions for each sex. The resulting estimate, expressed as number of females, is shown in Table V. Based on these estimates an allowance was calculated, using partial regression, leading to compensated exposure times: $x = 1.03035x_1 - .01212x_2 + .31226$, where x_1 is the original log-time, x_2 is the estimated number of females in the sample and x is the log of the compensated time. The latter is the time that would presumably be required for a sample of 50 oysters containing 28.37 females, which is the weighted average for the experiment, to reach the same degree of maturity as the given sample. This is presented in Figure 13, showing degree of maturity plotted against compensated exposure time, with a straight line of slope 4.343 fitted for each of the four temperatures. This fit is satisfactory after compensation for sex ratio: chi-square equals 30.8, with 22 degrees of freedom, which is just at the 10 per cent level of significance.

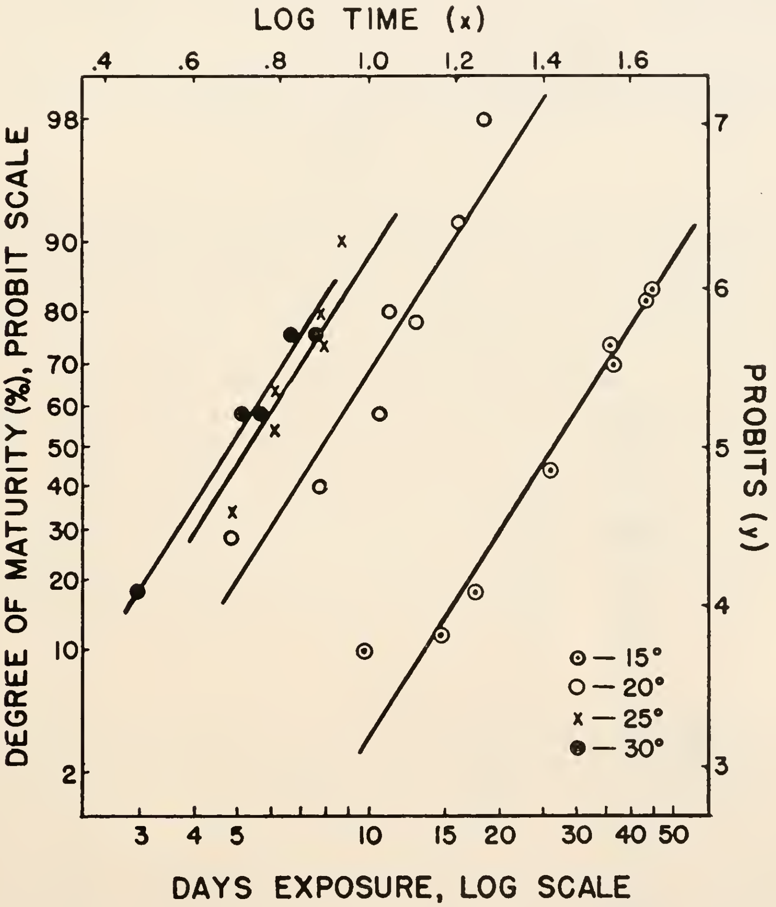


FIGURE 13. Degree of maturity (%) in samples of 50 oysters plotted against period of exposure to four experimental temperatures. Each period of exposure is compensated for the estimated sex ratio of the sample.

From the equation for each line, $y - \bar{y} = 4.343\ (x - \bar{x})$, where y is the probit of degree of maturity and $\bar{x}$ and $\bar{y}$ are the average values of x and y for a given temperature, the average time required for 50 per cent of oysters to develop mature gametes can be calculated for each temperature. This corresponds to the value of x when $y = 5$. For the four temperatures this average time was:

15.0° C. – 26.5 days	25.0° C. – 5.4 days
20.0° C. – 7.9 days	30.0° C. – 4.9 days

Their error is estimated as about 10 per cent. The lowest observation at 15.0° C. has been omitted in computing the value 26.5, because of its apparent discrepancy from other values at that temperature (Fig. 13).

We may fit these four points by a simple curve, from which average times needed for development of mature gametes can be estimated for intermediate temperatures (Fig. 14). The equation for the curve used is:

$$D = 4.8 + 4205e^{-.3554T}$$

where D is the average time needed, T is the temperature in degrees centigrade, and e is the base of the natural logarithms. The fit of this curve to the four established points is within their estimated error. This, however, is an empirical curve, and its theoretical significance, or its possible value for temperatures outside the range of the experiment, may be questioned.

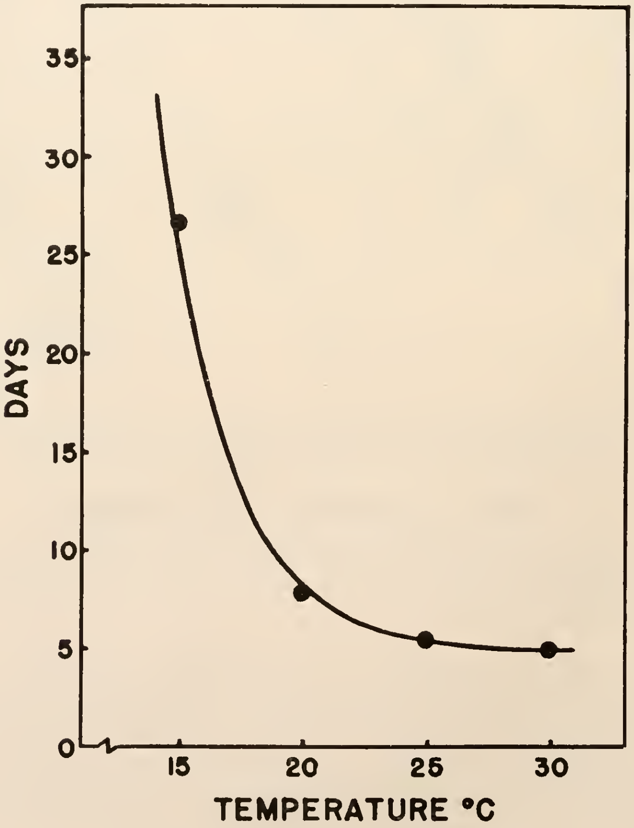


FIGURE 14. Average time needed for 50 per cent of an oyster population to develop mature gametes at different temperatures.

We have noticed that the quantity of glycogen contained by the oysters at the beginning of gonad development may control the quantity of spawn that will be produced. If the oysters are "poor," *i.e.*, containing little glycogen, they usually do not develop good gonads. For example, our attempts to condition for spawning in winter several groups of oysters shipped to us from the Florida waters of the Gulf of Mexico failed because those oysters were thin and watery, containing virtually no glycogen. Similar experiments with poor oysters from other bodies of water gave the same results.

In concluding this discussion it may be mentioned that the knowledge of the thermal history of the organisms prior to their exposure to the experimental conditions is important in interpreting the results of the experiment and in forming conclusions. Fry (1947), in his paper on the effects of the environment on animal activities, cites numerous examples where the thermal past of fishes reflects profoundly on their behavior under experimental conditions. It is possible, therefore, that if the groups of oysters used had been acclimated to a higher temperature than almost 0.0° C., which was our starting point, somewhat different results would have emerged from an experiment similar to ours. Furthermore, since it has been suggested that there are different physiological races among the population of *C. virginica* of our Atlantic coast, which require different temperatures for spawning (Stauber, 1950; Loosanoff and Nomejko, 1951), it is possible that our northern oysters, which are acclimated to comparatively low temperature, would, under the same experimental conditions, show a more rapid rate of gonad development and could be induced to spawn earlier than the oysters of other geographic regions, such as the Gulf of Mexico which, ecologically, maintains an entirely different temperature regime from Long Island Sound. Such relations to temperature conditions were found for several aquatic animals, including certain species of amphibians (Moore, 1939) where northern populations survived lower temperatures and bred earlier than the more southern groups.

Summary

1. The temperature of 10.0° C. was not high enough to induce normal gametogenic activity in most of the oysters. Even the most advanced individuals of this group showed only slight development of the gonad after exposure to this temperature for 35 days. Apparently this temperature is near the biological zero for this activity.

2. Ripening of gonads and spawning of oysters of both sexes were achieved at a temperature as low as 15.8° C.

3. Wide individual differences in the extent of gonad development among the individuals constituting the same groups were commonly found.

4. In all temperature groups, ranging from 15.0 to 30.0° C., the physiologically ripe gametes were generally formed earlier in the males than in the females.

5. At 15.0° C. the most advanced males contained a few ripe spermatozoa on the 10th day. Fertilizable eggs were found on the 20th day and spawning was induced by the 35th day. At 20.0° C. oysters with ripe spermatozoa and fertilizable eggs were found on the fifth day. Spawning was induced on the 10th and 13th days in males and females respectively. At 25.0° C. ripe spermatozoa and fertilizable eggs were found by the fifth day. Spawning was induced on the seventh day. At 30.0° C. ripe spermatozoa and a few fertilizable eggs were found three

days after the hibernating oysters were taken from their winter environment and placed at this temperature. Spawning was induced on the fifth day.

6. The average time at each of the experimental temperatures required for 50 per cent of the oysters to develop mature gametes was calculated to be:

15.0° C. – 26.5 days
20.0° C. – 7.9 days
25.0° C. – 5.4 days
30.0° C. – 4.9 days

To estimate the average time needed for development of mature gametes at temperatures intermediate to those given above, a simple curve is offered based on the equation:

$$D = 4.8 + 4205e^{-.3554T}$$

where D is the average time needed, T is the temperature, and e is the base of the natural logarithms.

7. The quantity of glycogen in the oysters at the beginning of gonad development may control the quantity of spawn produced.

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THE EFFECTS OF ETHYL ALCOHOL ON GROWTH AND RESPIRATION IN PELOMYXA CAROLINENSIS

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Among the first of comparatively few studies made on the effect of alcohol on unicellular organisms were those of Calkins and Lieb (1902) and Woodruff (1908). These workers used Paramecium and in general concluded that ethyl alcohol, in "moderate" concentration, acts as a stimulus in sustaining the vitality of these cells.

On the contrary, Matheny (1910) and Estabrook (1910) found no evidence that alcohol acts as a periodic or continued stimulus. In minute doses, 2% or less, it apparently has no effect on Paramecium while in doses of 3% or greater, it kills them. Daniel (1909) working with another genus, Stentor, found that ethyl alcohol causes an increase in growth rate. He also states that comparatively weak solutions of alcohol (1% or less) are ineffective, while a 4% solution causes early death.

More recently Bills (1924) observed that alcohol seems to restore starved paramecia to their former vitality. Loefer and Hall (1936) studied the effects of alcohol on several species of ciliates and flagellates. Of those studied, growth was accelerated in only two species, *Euglena gracilis* and *Euglena deses* (in 0.025 to 0.1% alcohol). Goldschmied-Hermann (1935) found that Paramecium dies in 3% ethyl alcohol. In concentrations of 1 to 2%, movements become sluggish and there is an increase in number of vacuoles and in the rhythmicity of their contractions. Three or four vacuoles were occasionally formed instead of the usual two.

Even fewer studies have been made on amoeboid cells and their reactions to alcohol. Brinley (1928) worked with Amoeba and found that 5% ethyl alcohol causes them to retract their pseudopods and take on a spherical form. The protoplasm becomes very fluid. Frederikse (1932) confirmed these results.

Daugherty (1937) investigated the effect of methyl (1.99 M), ethyl (1.028 M), propyl (0.467 M), butyl (0.164 M), and amyl (0.066 M) alcohols on *Amoeba proteus* and *Amoeba dubia*. In weak concentrations a liquefaction of the plasmagel occurs at first but with prolonged exposure to these concentrations the liquefied portion becomes gelated. In medium or strong concentrations of these alcohols, gelation of the plasmasol was observed.

Because of rather limited knowledge on the effects of alcohol on living cells and because of the ease with which Pelomyxa lends itself to studies of this type, the following investigations were carried out.

MATERIALS AND METHODS

The organism used in these investigations, *Pelomyxa carolinensis* Wilson (also referred to as *Chaos chaos*, *Chaos carolinensis* and *Amoeba carolinensis*), was grown in a solution developed by Pace and Belda (1944). The medium was composed of the following: K_2HPO_4 – 80 mg.; KH_2PO_4 – 80 mg.; $CaCl_2$ – 100 mg.; $Mg_3(PO_4)_2 \cdot 4H_2O$ – 2 mg.; and redistilled water to 1000 ml.

Paramecium caudatum was used as food for the pelomyxae; the latter are from 25 to 50 times as large as the former. The paramecia were grown in comparatively narrow jars (500 cc. capacity) containing the above medium plus hay infusion. A week or ten days after addition of the paramecia to a fresh culture they may be collected in abundance from the surface. Stacking dishes (finger bowls) of 10 cm. diameter, to which 150 cc. of pelomyxa solution or test solution were added, were used as culture chambers.

In ascertaining growth, both numbers of organisms and their volumes were taken into consideration. The volumes were measured by means of a volumescope (Chalkley, 1929; Belda, 1942). In these experiments the over-all average volume varies very little, except in the highest concentration of alcohol.

The rate of oxygen consumption was ascertained by means of a Barcroft-Warburg respirometer.

All the various dilutions were made from freshly prepared culture media and 100% ethyl alcohol, which was the only alcohol tested. The solutions were changed daily in an attempt to maintain the alcohol concentrations as constant as possible.

RESULTS

1. *Effect of alcohol on growth in Pelomyxa*

Ethyl alcohol was diluted with the culture solution, as previously described, to the following concentrations: 0.001 *M*, 0.005 *M*, 0.01 *M*, 0.05 *M*, 0.1 *M* and 0.5 *M*. In each experiment, three culture dishes were used for each concentration and 100 ml. of solution containing 25 pelomyxae were placed in each as well as in the control cultures (no alcohol). Each culture received one ml. of washed and concentrated paramecia every second day thereafter. The stacking dishes were sealed with Lubriseal to prevent possible loss of alcohol by diffusion from the culture.

The results of four experiments are presented in Table I. It is evident that 0.5 *M* alcohol is toxic to Pelomyxa. In every experiment there was a decrease in the number of organisms placed in this concentration and in Experiment 4 they died out altogether by the eighth day. In 0.005 *M* alcohol, growth is 29% greater than in the control cultures; it is also greater in 0.01 *M* and 0.05 *M* alcohol (12% greater in both) but higher concentrations than these have a retarding effect if they do not actually kill the organisms.

2. *Effect of alcohol on structure, size and activity of Pelomyxa and upon digestion of food*

Other characteristics, aside from growth, are also affected by alcohol. For example, at the end of 24 hours in solutions containing 0.5 *M* alcohol, only rarely is a pelomyxa found attached to the substratum. However, in the control and in the 0.001 *M* and 0.005 *M* alcohol solutions, they seemed to be firmly attached.

The organisms are also quite active in these solutions although in 0.01 *M*, 0.005 *M*, and 0.001 *M* alcohol they are much less active and even slight jarring of the culture dish will easily dislodge them if they are attached. In spite of these differ-

TABLE I

Growth of Pelomyxa in various concentrations of ethyl alcohol

Experiment No.	Molar concentration of alcohol	No. of pelomyxae on days (as designated) following inoculation					Total increase
		2	4	6	8	10	
1	0	34	39	54	127	208	183
	0.001	32	40	52	109	173	148
	0.005	29	41	62	145	276	251
	0.01	32	38	50	131	219	194
	0.05	30	44	65	170	252	227
	0.1	26	28	32	32	34	9
	0.5	23	22	22	18	10	−15
2	0	30	34	51	107	151	126
	0.001	33	46	67	116	192	167
	0.005	36	47	71	128	203	178
	0.01	34	48	63	136	224	199
	0.05	34	45	75	141	211	186
	0.1	29	37	49	72	108	83
	0.5	26	28	26	15	5	−20
3	0	28	34	84	177	262	237
	0.001	28	35	76	183	246	221
	0.005	29	36	99	265	342	317
	0.01	29	36	77	231	321	296
	0.05	26	32	99	257	328	303
	0.1	27	29	72	138	272	247
	0.5	26	24	22	19	18	−7
4	0	35	52	74	199	271	246
	0.001	27	49	74	177	253	228
	0.005	33	41	89	208	305	280
	0.01	29	44	74	162	224	199
	0.05	30	42	68	139	204	179
	0.1	25	25	32	47	91	66
	0.5	18	10	6	0	0	−25
Average for all experiments. (32 cultures for each concentration)	0	32	40	88	152	223	198
	0.001	30	42	87	148	218	193
	0.005	32	41	80	186	281	256
	0.01	31	41	88	185	247	222
	0.05	30	41	77	177	248	223
	0.1	27	30	48	72	126	101
	0.5	23	21	19	13	8	−17

Eight cultures were used for each concentration in each experiment; 25 pelomyxae were added to each culture; counts were made every two days; temperature, 25° ± 1° C.

ences between the experimental and control animals growth is greater in 0.01 *M* and 0.05 *M* alcohol solutions.

Pelomyxae exposed to high concentrations of alcohol, especially 0.5 *M*, are very inactive and appear to have a narrow solated area. The pseudopods are short and rounded. Very few food vacuoles are found, although numerous dead paramecia are scattered over the bottom of the dish. In Figure 1, a typical normal pelomyxa is compared with one grown in 0.5 *M* alcohol solution.

One of the most striking structural changes is found in the hyaline layer, which in normal organisms is a narrow clear area (except at the tips of pseudopods, where it is quite pronounced) found immediately beneath the plasmalemma. In pelomyxae exposed to 0.5 *M* alcohol for 24 hours, the hyaline layer becomes greatly enlarged and well-defined in all parts of the organism. The area becomes more noticeable from day to day, and appears to be most noticeable on about the fourth or fifth day of exposure. The increase in depth of the hyaline layer is noticed in all concentrations from 0.05 *M* and higher. Another noticeable change is in the decrease in size of the pelomyxae in the high alcohol concentrations. After 8 days in 0.5 *M* alcohol, they are about 1/10 their original size. This is illustrated well in the figure.

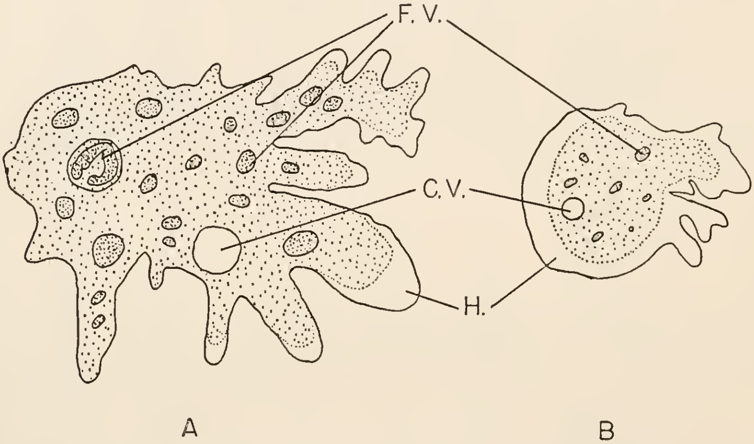

FIGURE 1. Camera lucida sketches of (A) a typical pelomyxa from a well-balanced culture solution without alcohol, and (B) one from the same solution containing alcohol in 0.5 *M* concentration. F. V., food vacuole; C. V., contractile vacuole; H., hyaline area.

The rate of digestion of food organisms is also retarded in high concentrations of alcohol. In the controls and in the 0.001 *M* to 0.01 *M* alcohol solutions, most of the food vacuoles contain only fragments of paramecia after 24 hours while in higher concentrations rather large fragments are found even after 5 days. In the 0.5 *M* alcohol solution the food vacuoles are carried over from the time of ingestion before exposure to alcohol; no paramecia are ingested by the pelomyxae after the latter are exposed to this concentration.

3. *Effect of alcohol on respiration in Pelomyxa*

The Barcroft-Warburg respirometer was used to ascertain oxygen consumption. This was done by means of the "direct method" as described by Pace and Belda (1944). Tests were made on pelomyxae in solutions without alcohol, and in solutions containing 0.005 *M* and 0.01 *M* alcohol. For each concentration, two sets of tests were run: in one, the organisms were introduced into the alcohol solution just preceding the test; in the other, they were placed in the solutions 24 hours preceding the test. All the organisms were "well-fed" as described by Pace and Belda (1944).

To each Warburg flask, 500 pelomyxae were added. These were approximately uniform in size. The volumes of 30 pelomyxae selected at random were ascertained by means of a volumescope (Pace and Belda, 1944) before and after the tests and found to approximate 35,000 mm.^{3} per million organisms. The results are presented in Table II. The duration of each experiment varied between 5 and 9 hours. The oxygen consumption was ascertained in mm.^{3} per hour per million organisms as well as in mm.^{3} per hour per mm.^{3} protoplasm.

TABLE II

Oxygen consumption in Pelomyxa carolinensis in different concentrations of alcohol as compared to that in the absence of alcohol

Concentration of alcohol	Number of tests	Duration of experiment	Average rate of O^2 consumption in mm.^{3} per hr. per 10^6	Average O_2 consumption in mm.^{3} per hour per mm.^{3} cell substance
Organisms placed in test solutions just preceding test				
0 (control)	9	5–6 hrs.	9,800	0.280
0.005 M	9	5–6 hrs.	16,300	0.465
0 (control)	9	5–9 hrs.	7,200	0.205
0.01 *M*	9	5–9 hrs.	14,800	0.422
Organisms placed in solutions 24 hours preceding test				
0 (control)	9	5–7 hrs.	10,700	0.305
0.005 *M*	9	5–7 hrs.	23,500	0.671
0 (control)	9	5–7 hrs.	5,000	0.143
0.01 *M*	9	5–7 hrs.	20,900	0.568

Temperature, 25° ± 1° C.; average volume of 1 million pelomyxae, about 35,000 mm^3.

In both test solutions, the oxygen consumption is greater than in the control solutions which had no alcohol present. These results are therefore of a similar nature to those obtained in the growth studies in which growth increases appreciably in both these concentrations. In fact, the accelerating effect of alcohol is much more pronounced in the respiratory studies. The effect is even greater if the organisms are placed in the alcohol solutions for 24 hours before the tests are run.

DISCUSSION

Studies made upon ethyl alcohol and its effects on unicellular animals are not very extensive. In the earlier work cited previously, most of the tests were carried out with Paramecium as experimental animal although some observations were made upon amoeboid organisms. A comparison of the results seems to indicate that the amoeboid cells react to alcohol in a manner somewhat similar to paramecia which were usually killed by concentrations around 3% (0.75 *M*).

In the tests reported here, in which observations were made at various concentrations, most of the pelomyxae that had been put into 0.5 *M* (2.3%) alcohol solutions were dead at the end of 10 days; on the average, only 8 out of every 25 organisms were still alive.

In lower concentrations (0.005 *M* and 0.01 *M* alcohol) there was an actual increase in growth of these organisms. Associated with this growth increase is a very noticeable acceleration in oxygen consumption of the pelomyxae in the same concentrations of alcohol. This would suggest that the energy released on the oxidation of alcohol is available for certain processes in Pelomyxa.

Evidently much of the energy produced by this means is wasted; at least this is true if growth can be used as a measure for assimilative metabolism. For example, the optimum alcohol concentration for growth was found to be 0.005 *M,* in which there were produced on the average 281 organisms from 25 pelomyxae over a period of 10 days. This is a total increase of 256 organisms, and represents an increase of 30% over the control organisms without alcohol. On the other hand, in this same concentration of alcohol, there was a much higher percentage increase in oxygen consumption; a 66% increase in the organisms that were not adjusted and 119% in those that had lived in the alcohol for 24 hours before oxygen consumption was ascertained.

SUMMARY

1. Specimens of *Pelomyxa carolinensis* were exposed to various concentrations of ethyl alcohol and observations made upon their rate of growth and respiration. The following concentrations were tested: 0, 0.001 *M,* 0.005 *M,* 0.01 *M,* 0.05 *M,* 0.1 *M,* and 0.5 *M* and 1.0 *M*.

2. In 1.0 *M* (4.6%) alcohol all the organisms were dead within 24 hours. In 0.5 *M* (2.3%), although most of them died early, some lived for a 10-day period.

3. Growth was accelerated in 0.005 *M,* 0.01 *M,* and 0.05 *M* alcohol; the greatest acceleration was a 30% increase over the control in 0.005 *M*.

4. In the higher concentrations of alcohol (0.5 *M* and 0.1 *M*) the pelomyxae do not feed and show considerable decrease in size; the hyaline layer becomes very large.

5. Rate of respiration was found to be much greater in 0.01 *M* and 0.005 *M* alcohol than in the controls without alcohol. It was greatest in 0.01 *M,* especially when the organisms were put into the alcohol solution for 24 hours before the tests were run, in which case respiration was 318% greater than in the controls.

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THE REGULATION OF SPONTANEOUS STRUCTURAL ANOMALIES IN PELMATOHYDRA OLIGACTIS

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It is necessary to specify the exact meaning of the word when employing the term regulation. Driesch (1901) defined it as a process or a change in a process occurring in a living organism, by means of which a disturbance of its pre-existing normal condition is wholly or in part compensated and the normal condition is re-established. Roux described regulation as a compensation of disturbance, *i.e.,* the return of the disturbed organism to a type or norm. Roux emphasized the part of heredity in the control of regulatory processes while Driesch was inclined to invoke extrabiological metaphysical controls. Morgan (1898), who had been working in the field of regeneration, coined the term morphallaxis to include the remodelling of old tissue into new forms. He later (1907) extended the use of the term to include re-differentiation and regeneration. Driesch used the terms as a synonym for restitution by redifferentiation. Morgan (1907) assumed that the influence of the formed parts upon the unformed parts during their development was a pressure or some kind of a response to a pressure. Child (1906) after working extensively on regeneration of Planaria and other forms came to the conclusion that living organisms are in a continual state of regulation physiologically and that no particular group of processes could be separated as formative as compared with those which, in the normal adult animal, maintained it in a state of partial equilibrium. Jennings (1905) applied the concept of regulation to the field of animal behavior and concluded that all animal behavior, in its more complex aspects as well as its simpler ones, is attributable to regulation.

Experimental embryologists point out (Weiss, 1939) that the course of embryology is rather fixed in animals with a mosaic type of development and that experiments involving interference with the regular course of development are not very productive. Other animals in which the course of development is not firmly fixed at an early stage, referred to as regulative types, will sometimes rearrange the cellular components in excised parts of an embryo and form one or more perfect embryos from material originally destined to form only a part of an embryo. Spontaneous abnormalities in embryos may rearrange their parts so as to form a normal embryo. The term regulation is used to indicate the processes by which the embryos undergoing experimental interference or embryos having spontaneous anomalies rearrange their materials so as to produce perfect whole individuals. It is assumed that the processes and the controls of the processes are identical with those which guide development in the course of normal development. Presumably the regulative mechanism continues to exist in fully formed individuals although, under normal conditions, there is no opportunity for the mechanism to be exhibited.

Curtis defines regulation as all of the processes by which the organism returns to the physiological and morphological type that characterizes the species after the type has been disturbed. Regeneration is considered to be a form or phase of regulation. Morphallaxis is defined as the process by which proportions are regained after disturbance.

Needham (1950) confines the term morphallaxis to morphological modification or regeneration without the formation of new cells. Regulation is described as the power of continuing normal or approximately normal development or regeneration in spite of experimental interference. The processes operate in all normal embroys and are a part of the sum total of processes whereby the organism is to some extent rendered independent of its environment.

This survey of the uses of the terms regulation and morphallaxis indicates that there are considerable differences in the interpretations of their meanings. The writer will use the term regulation in much the same sense as it is employed by Child (1924). He points out that in the reconstitution of a whole from a part during regeneration, the course of development in a reconstituted piece of an animal is not normal (as compared with the course of development which produced it in the first place) but the results as regards form and structure are normal. The over-all result of regulation will be regarded as a kind of holding action by which an aberrant specimen is returned to the parental type. The term regulation will be used strictly, as it is by Hyman (1928), for the series of spontaneous changes by which an anomalous specimen is returned to the normal species and clonal type. None of the anomalies to be described arose because of experimental interference.

No attempt will be made to determine the character of the regulative mechanism but it will be re-emphasized that the regulative course through which morphological anomalies are returned to normal types is very different from that through which the normal type was produced in the first place. The regulative processes occur in the presence of such mature states as budding, depression, formation of sexual organs, ect. The influences of these states upon regulation will be given particular attention.

Material and Methods

The specimens of *Pelmatohydra oligactis* used in this study were all the progeny of a single male specimen with 5 tentacles. Some of the observations on regulation were incidental to a study of the daily rate of reproduction and the reproductive potential of a single clone (Turner, 1950). In most of the cases of aberrancy in structure, the individuals concerned were members of a pedigreed strain which had been under observation for many months and for which a complete record had been kept. A large discard pool for the clone was kept in a 50-gallon tank. Specimens which were not studied in individual cultures were placed in this pool and given maximal feeding. Thousands of specimens were produced in the pool and the pool was examined from time to time for aberrant specimens. When aberrant specimens were discovered they were removed from the pool and cultured individually so that continuous individual records could be kept.

Records of pedigreed specimens as well as specimens selected and cultured from the pool consisted of notations on tentacle number, formation of spermaries, number and rate of bud production, duration of processes concerned in regulation (absorption of parts, fusions, divisions, migration of specific parts, etc.), and the cycles of depression and recovery.

Longitudinal Fission

There are a number of temporary conditions which, when considered in a single stage, give the appearance of longitudinal fission. It is necessary, therefore, to follow suspected cases through to a conclusion before they can be classified definitely as cases of apico-basal fission. All the eight cases described here occurred in a single clone, in either pedigreed stock or in a discard pool of the clone. All were put under daily observation and records were kept until fission was complete or the specimens died. Five of the cases were found in specimens of which daily records had been kept and three were discovered in the discard pool. The complete records have made it possible to determine the total length of time occupied in fission and the relative lengths of time required for fission of the different parts of the body. The relation of fission to spermary formation and to periods of active budding and depressions was also observed.

All of the five cases which originated in pedigreed stock under observation began in buds which had not yet become detached. In all of these cases the parent animals had just emerged from a state of depression and the attached buds had gone through the depression with the parents. One of the three cases discovered in the discard pool was in an incipient stage of development as indicated by the half-developed tentacles. No instance was observed in old fully developed specimens in which a bifurcated condition of the apex resulted later in longitudinal fission. Numerous cases of a bifurcated apex in old specimens have been observed, but the return to a normal state was attained by other methods of regulation.

Two of the cases of longitudinal fission were recorded as follows:

Specimen 1. The specimen was observed as a partially developed bud with a bifurcated apex. Development was completed by the next day when the bud separated from the parent. Six days later a bud appeared upon the newly formed bifurcated individual at a point well below the region of the bifurcation. Fission was slow, requiring 38 days to reach the budding zone. During the 38-day period 21 buds were given off. The fission of the single budding zone required five days during which one of the apical members gave off seven buds and the other gave off eight buds. Eight days were required for the division of the stalk below the budding zone and of the base. During the last eight days of fission the specimen was in a state of depression during which it did not feed and gave off no buds.

Specimen 2. This specimen was observed first as a bifurcated bud which was about to be separated from the parent. After separation from the parent, which occurred 16 hours after the first observation, the specimen began budding for itself. The first bud appeared just below the point of bifurcation three days after separation from the parent. By the end of 33 days fission had reached the budding zone and eight days were required for the division of the budding zone. The stalk below the budding zone and the base was completely divided in another nine days. A total of 19 buds was given off before fission reached the budding zone. During the division of the budding zone, the stalk and the base, nine buds were given off by one member and 11 buds by the other. Spermaries formed upon the undivided and also the divided portion of the specimen 17 days after it had separated from the parent and persisted for seven days: During the period of observation two depressions occurred, one lasting for four and the other for six days. The state of depression appeared to have no effect upon the progress of the process of fission. Fission of the stalk and of the base was completed during a period of active budding.

There was some variation in the division time of the other three specimens recorded from pedigreed stock and in the three specimens discovered in the discard pool. The time required for the first appearance of buds on bifurcated specimens after they had separated from their parents varied from three to seven days. Division from the hypostome down to the budding zone required from 27 to 41 days. The time involved in the division of the budding zone was brief and varied from 5 to 11 days. The division of the stalk was relatively rapid and in no case required more than six days. Fission lagged upon reaching the base and in the most extreme case 12 days were required for its complete division.

One aberrant specimen should be described here. The specimen separated from the parent as a bud which was almost completely developed. The apical end was bifurcated but one hypostome was larger and better developed than the other. The specimen fed for one day only, through the better developed mouth and hypostome, and then went into a prolonged depression from which it did not recover. It became inactive, the tentacles became short and bulbous and there was no formation of buds or sexual bodies. However, the process of longitudinal fission continued and was completed seven days after separation from the parent. Fission resulted in the formation of two individuals of unequal size, both of which disintegrated two days after fission was complete.

Two buds which had deep divisions in the hypostomal regions were permitted to separate from the parent animals and they were then submitted to complete starvation. Both of the specimens died in less than a month and during this time longitudinal division did not progress.

Chang, Hsieh and Liu (1952) found that fission occurred in a ratio of about 1/1000 to normal buds. They found that buds with twin apical ends might arise either through division or by fusion of specimens located in close proximity on the parental stock. They noted the surprising situation also of complete division of buds before detachment from the parent. Neither of these phenomena were observed in the stock used by the writer.

Summary: A summary of the facts relating to longitudinal fission indicate that: (1) the bifurcated condition of the apex which initiates longitudinal fission originates most commonly, and possibly exclusively, in buds which are in the latter stages of development and are still attached to the parent. A bifurcated condition of the apex is fairly common in mature specimens recovering from a physiological depression but none of the specimens observed in this condition resolved the abnormality by undergoing longitudinal fission. (2) In most cases of longitudinal fission the hypostome had been divided previously into two equal parts to initiate fission. In exceptional cases hypostomes which had been divided unequally initiated complete fission. (3) The early phases of fission involving division down to the budding zone are accomplished in a relatively short time (5 to 11 days). (4) The division of the stalk and the base requires about eight days ordinarily but one specimen required only four days and another 16 days. (5) The progress of longitudinal fission does not seem to be retarded by a state of depression in a specimen. (6) A state of active budding does not appear to affect the rate of fission. (7)

Spermaries may be formed, come to maturity and disappear without changing the rate of fission. (8) Complete starvation appears to stop the progress of longitudinal fission.

Reduction of Supernumerary Hypostomes and Tentacles

Sixteen specimens were observed among approximately 3500 pedigreed individuals of a single clone in which at some period the hypostome was divided and reduced to the single state again by fusion. While this appears to represent a high incidence for the condition, it is believed, for reasons that will be mentioned later, that cases in which the hypostome undergoes slight division and subsequent fusion are quite common. Two of the 16 cases observed occurred in buds which separated from the parents with slight but clear cut divisions of the hypostome, two mouths and supernumerary tentacles and later regulated into single individuals by fusion of the hypostomes and tentacles. In the two cases occurring in buds the divided hypostomes were composed of unequal parts. The remaining 14 cases occurred among old specimens. All of the cases were observed for several weeks after their discovery. A search of the discard pool of the same clone revealed other specimens with divided hypostomes but these were not studied further.

All of the instances of divided hypostomes in old individuals occurred in specimens which, after a period of reproduction by budding, had gone into a state of depression characterized by inactivity, cessation of budding and feeding and a partial disintegration of the hypostome and tentacles and were recovering from the depression. Periods of depression were of two types, one lasting from one to four days and the other from 6 to 12 days. The most pronounced disintegration occurred in specimens undergoing the longer periods of depression. The divided hypostomes occurred in specimens which were regenerating the hypostomes after depression and partial disintegration. In every case, supernumerary tentacles appeared. When the hypostome was divided equally and two functioning mouths were present the number of tentacles was doubled so that 10 tentacles were present. When the hypostome was unequally divided and two mouths were present but only one was functional, the number of supernumerary tentacles was less. It would appear that the condition of a divided hypostome should result in longitudinal fission but no such fission was observed in these old specimens. Instead, a fusion occurred which reduced the doubled condition of the mouth and hypostome to a normal condition within a few days. The reduction of the supernumerary tentacles by fusion to the 5 characteristic of the clone took much longer.

In rare instances, divided hypostomes in buds became fused instead of initiating longitudinal fission. A bud with a divided hypostome was discovered on an old individual bearing three other buds. The parental individual was in the midst of an accelerated reproductive period. The partially divided bud was second from the oldest of the attached buds and since no period of depression had occurred for several days, it is obvious that the bifurcated condition of the bud, unlike that in mature individuals, did not arise because of a distributed growth rate produced by a depression and a recovery from the depression. The buds just older and younger than the partially divided one were normal in every way and it is not possible reasonably to assign any recognized state of the bud or of the parent as the cause of the bifurcation. The partially divided individual was examined daily for 52 days after it was detached from the parent. The divided hypostome was fusing before the bud became detached and it was completely fused within three days of detachment. Three short supernumerary tentacles, which had arisen while the hypostome was divided, were irregularly disposed on the hypostome, two outside the regular ring of 5 and one inside the ring near the mouth. This small tentacle near the mouth was absorbed within two days. The other supernumerary tentacles were completely absorbed within 18 days without fusing with any of the tentacles in the regular ring of 5. The specimen gave rise to 34 buds during the next 52 days, each bud bearing 4 or 5 tentacles when detached and 5 tentacles when mature.

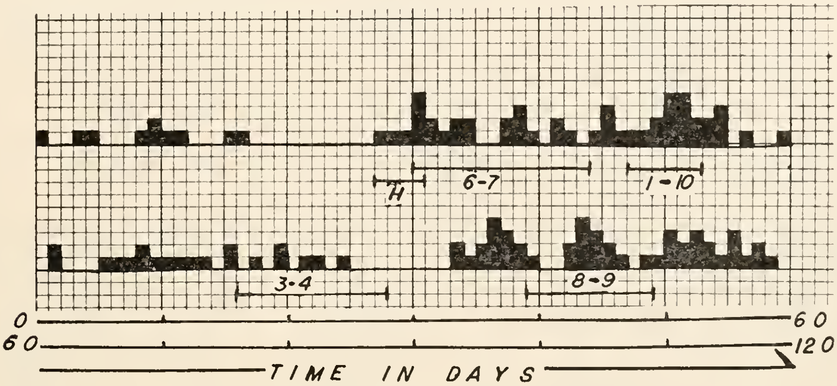

Diagram 1. Block diagram showing relation of reproduction by budding and of depressions in Specimen 5.1.10 to regulatory processes. Blocks represent new buds produced per day. H represents period of fusion of divided hypostome; 6–7, 1–10, 3–4, 8–9 represent periods of fusion of tentacles indicated by the numbers.

The fairly typical formation of a divided hypostome and the subsequent regulation of the aberrant condition by fusion of the hypostome and tentacle in a mature specimen is shown in Diagram 1 and in Figures 12 to 18. In Diagram 1 blocks represent the number of new buds formed per day. The largest number of buds formed in one day is four. It will be noted that periods of rapid bud production are interspersed with periods of one to three days during which specimens produce no buds. Two prolonged periods of depression occur between the 18th and 27th day and the 85th and 93rd day. The four-day period of time indicated by H is the period during which the divided hypostome fused and the periods of time indicated by 6–7, 1–10, 3–4, and 8–9 are those during which the numbered tentacles were fusing. The 120 day history of the specimen is as follows: During the first 17 days of observation, 10 buds were produced. with intervals of rest of two to three days between periods of budding. The parent animal had 5 tentacles (Fig. 12) as did the buds. A period of depression occurred on the 17th day (Diag. 1), during which the mouth disappeared, part of the hypostome disintegrated and the tentacles were reduced to short stubs (Fig. 13). The period of depression was followed by rapid recovery involving regeneration. During regeneration (Diag. 1, day 23 to 26) two hypostomes and two mouths were formed. Nine tentacles of the usual length were formed and one short tentacle (Fig. 14, 1). Fusion of the two hypostomes (Figs. 14, 15) occupied four days (Diag 1). Before fusion of the

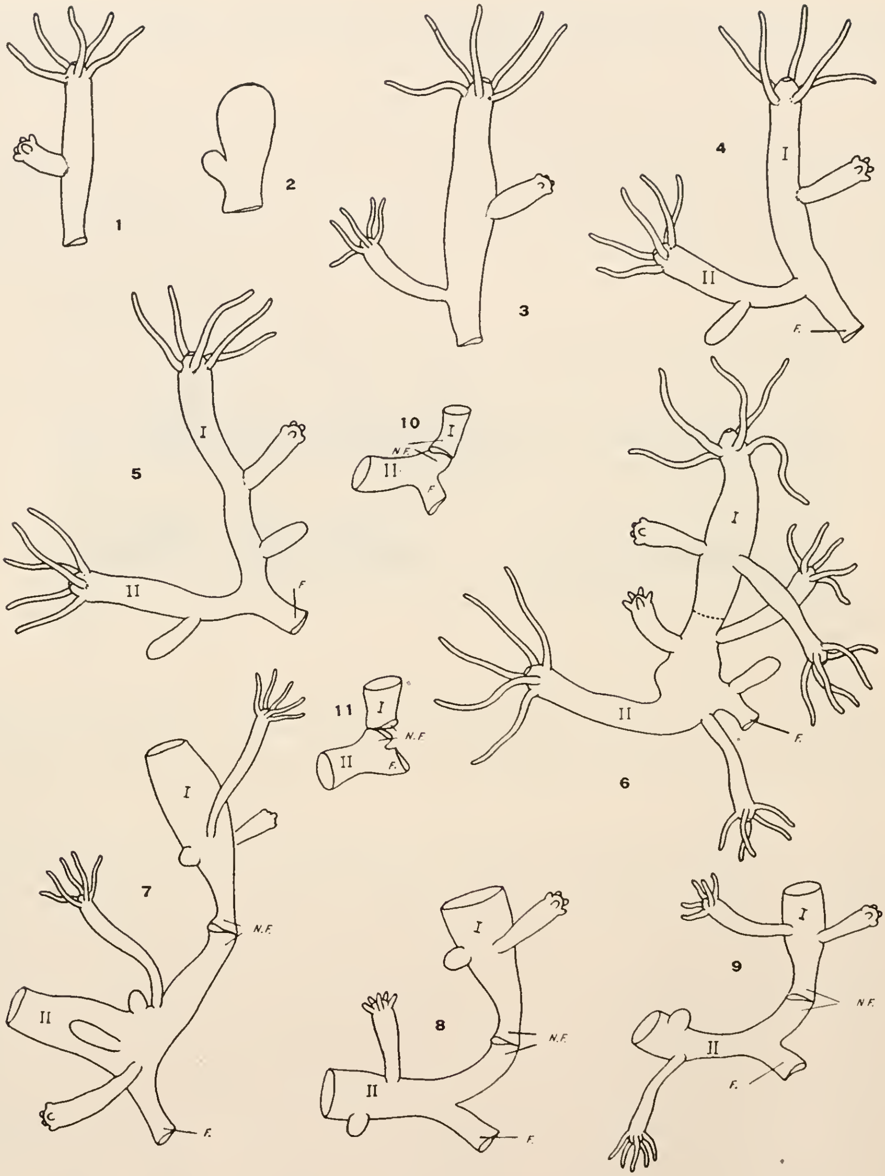
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Plate 1

Figure 1. Specimen of male *Pelmatohydra oligactis* with attached bud prior to period of depression. Following figures indicate progress in the same specimen.

Figure 2. The specimen during depression.

Figure 3. Emergence from depression; regeneration of apical parts by parent and bud; shifting of bud toward base of parent; resumption of budding by parent.

Figure 4. Failure of old bud to detach (II); old bud producing bud of its own.

Figure 5. Budding zone of parent (I) elongating; both parent (I) and old retained bud (II) budding rapidly to form colony.

hypostome was complete, the specimen was budding vigorously (Diag. 1, days 27–35) and tentacles no. 6 and 7 were fusing, beginning at the base and proceeding apically. The fusion required 14 days. A fusion of tentacles 1 and 10 began on the 47th day and was completed by the 53rd day (Fig. 16) (Diag. 1, 1–10). Figure 17 represents the specimen on the 75th day. Tentacles 3 and 4 fused between the 76th and the 88th day (Diag. 1, 3–4) (Fig. 18) and tentacles 8 and 9 fused between the 99th and the 109th day (Diag. 1, 8–9). A typical long depression and a recovery occurred between the 85th and the 93rd days (Diag. 1) but no abnormalities arose in connection with this period. Tentacles 3 and 4, which had been fusing for 9 days before the beginning of the depression, completed their fusion during the first three days of the depression.

Specimens in clone No. 5, upon which the most extensive observations were made, had 5 or 6 tentacles in mature individuals but occasionally pedigreed specimens were found in temporary states with as many as 10 tentacles. All of the cases occurring in pedigreed specimens reduced the number of supernumerary tentacles by fusion. In the last case described under "fusion of divided hypostomes" 80 days were required to reduce the number of tentacles from 10 to 6. In some other instances in the pedigreed stock, a few supernumerary and poorly formed tentacles appeared after depressions and recovery from depressions in individuals which did not have divided hypostomes. Specimens with more than 5 tentacles were observed in the discard pool of this clone rather frequently. It occurred to the writer, as a result of these observations, that the tentacle number for a clone might be more definitive than would become apparent by a single examination of a large number of individuals. A single examination would indicate a considerable range in number and it would be reasonable to assume this to be the natural range of variation. However, if it is a fact that a narrow definitive range of tentacle number exists in a clone, then all of the specimens with a tentacle number larger than the definitive number could be in a state of regulation during which the definitive tentacle number would be restored.

The hypothesis that large numbers of hydra are normally in a state of regulation as regards tentacle numbers and that nearly all specimens with supernumerary tentacles should be included among the regulating specimens can be tested by several different types of observations. (1) The tentacle number can be recorded for all of the buds of a single parent animal which itself is the product of a line with a definitive number of tentacles. The production only of buds with the same number of tentacles as the parent would indicate a maintenance within the clone of the definitive number. (2) Departures by buds from the tentacle number characteristic of the parent can be associated with events, if any, in the life history of the clone producing the bud. (3) Discard pools of clones with a definite number

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FIGURE 6. Appearance of transverse diaphragm (dotted line) in the budding zone of parent (I); fusing and adjustment of budding zones of individuals I and II.

FIGURE 7. Apical parts not shown; further progress of tranverse division and differentiation of new feet (N.F.) at site of diaphragm.

FIGURE 8. Apical parts not shown; migration of region of old foot (F.) toward site of transverse division (N.F.).

FIGURE 9. Further migration of old foot; both individuals (I and II) budding actively.

FIGURES 10 and 11. Completion of migration of old foot to fuse with nearest of new feet; budding is active but bases only are shown.

of tentacles can be examined from time to time and if specimens with supernumerary tentacles appear they can be isolated and observed. (4) A census of tentacle number can be taken of all the specimens in a discard pool from time to time to determine whether the total range of variation has changed. (5) Specimens with tentacle numbers larger than ordinary, from sources other than discard pools, can be isolated and observed. All of these types of observations have been used and the results have supported the hypothesis that the degree of variation in tentacle number is not great and that the specimens which vary much from the clonal number are in a state of regulation.

The most complete record of a specimen which reduced the tentacle number by fusion has been described (Figs. 12 to 18; Diag. 1) under the section on "fusion of divided hypostomes." Descriptions of some other cases offering a number of variations follow.

Specimen 5.1.3 from a clone with a characteristic tentacle number of 5 had 7 tentacles after a two day depression and a one day recovery. The specimen had previously given rise to 107 buds at the approximate rate of 1.14 buds per day. During the short period of depression the hypostome degenerated and the tentacles became short and bulbous. On recovery, there were 5 tentacles in the normal position and two short tentacles on the hypostome within the ring of 5. As all of the tentacles increased in length, the short tentacles began fusing at the bases with the nearest tentacles within the ring and within five days the fusion was complete.

Specimen with 7 tentacles. This specimen arose from a bud with a divided hypostome in non-pedigreed stock. After the bud separated from the parent the divided hypostome fused into a single one with 8 tentacles of irregular lengths placed around the hypostome in an irregular row. The shortest tentacle was absorbed by the hypostome within two days without fusing with any other tentacles and the remaining 7 tentacles became adjusted so as to form a perfect ring about the hypostome. The 7 tentacles became equalized in length by differential growth. The specimen lived for 17 days during which it gave off 31 buds, 25 of the buds having 5 tentacles and 11 buds having 4 tentacles. These latter individuals developed 5 tentacles when mature.

Specimen with 9 tentacles. This pedigreed individual on recovery from a depression had 9 tentacles. Two tentacles were fused (or forked) near the ends, two were located upon the hypostome near the mouth and five were normal and in normal positions. The forked pair became single by fusion within 24 hours. The two short centrally placed tentacles first fused with each other and were then ab-

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FIGURE 12. Specimen 5.1.10 in normal active condition. All figures are of the same specimen.

FIGURE. 13. Partial disintegration during period of physiological depression.

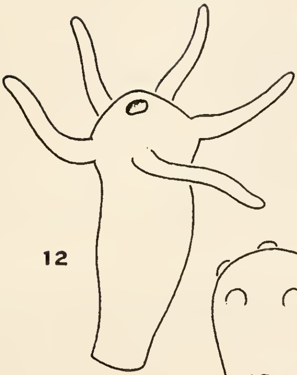
FIGURE 14. Condition following depression and recovery; two mouths, two hypostomes and supernumerary tentacles.

FIGURE 15. Fusion of two hypostomes in progress; tentacles no. 6 and no. 7 fusing.

FIGURE 16. Two hypostomes are completely fused; tentacles 6 and 7 are completely fused; tentacles 1 and 10 are in process of fusing (22 days after recovery from depression).

FIGURE 17. Fusion of tentacles 1 and 10 complete.

FIGURE 18. (53 days after recovery from depression). Tentacles 3 and 4 are fusing. (Tentacles 8 and 9 began to fuse 72 days after recovery from depression and were completely fused 11 days later.)



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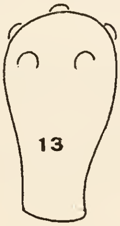

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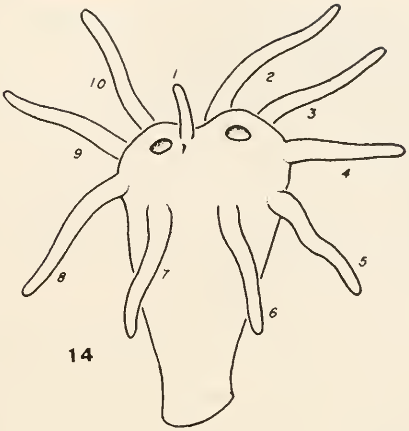
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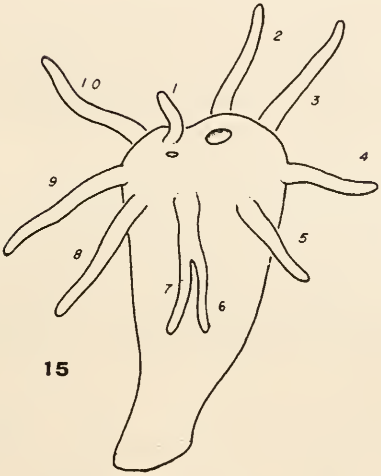
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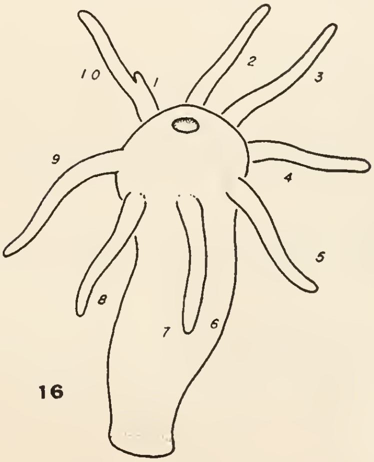
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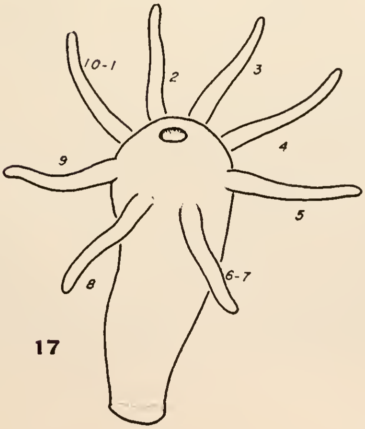
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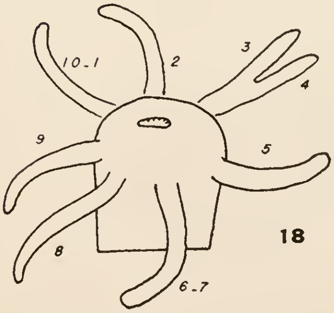
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Plate 2

sorbed by the hypostome. The absorption required 8 days. Buds arising from this individual had 5 tentacles.

Pedigreed specimen with 5 tentacles. This specimen from the discard pool was observed for 54 days and had 96 buds. Two buds had 6 tentacles and all of the others had 5.

Pedigreed specimen with 6 tentacles. In a period of 13 days, this specimen produced 16 buds of which 3 had 6 tentacles and 13 had 5.

Pedigreed specimen with 7 tentacles. No fusion of tentacles was observed in this specimen which was observed for 15 days but of the 20 buds produced, 15 had 5 tentacles and 5 had 6 tentacles.

Discard pools of 5-tentacle strain. One pool had a total of 104 specimens of which 99 had 5 tentacles, three had 6 tentacles and two had 7 tentacles. However, one of the three specimens bearing 6 tentacles and one of the two specimens bearing 7 tentacles had each a pair of tentacles fusing into single tentacles. In another pool examined just after the entire pool had undergone a depression and a recovery, the tentacle numbers were observed as follows: 236 had 5 tentacles; 38 had 6 tentacles; three had 7 tentacles; one had 8 tentacles; and one had 9. Three specimens with pairs of fusing tentacles were observed in th 6-tentacle group and one specimen with a pair of fusing tentacles occurred in the 9-tentacle specimen.

In rarer instances, specimens emerge from depression with one tentacle branched or split. The branching may be single or multiple. All such specimens observed resolved the condition by progressively absorbing the branches until a single tentacle remained.

Chang, Hsieh and Liu (1952) observed that tentacles of *Pelmatohydra* varied in number normally from 5 to 10 and that by treatment with phosphate, specimens with as many as 17 tentacles could be produced. In the opinion of these authors longitudinal division, which is frequently associated with the possession by a specimen of supernumerary tentacles, is caused by the excess tentacles. It seems to this writer that the supernumerary tentacles and the bifurcated condition of the hypostome might both arise because of loss of control of the growth pattern during a depression.

Summary: The conditions arising in the apical ends of specimens during depressions are disintegration of the hypostome to some degree and loss of partial disintegration of the mouth and tentacles. During recovery a temporary loss of factors controlling normal morphogenesis may result in double hypostomes with a supernumerary mouth and, after fusion of double hypostomes, supernumerary tentacles may be present. The regulatory processes by which individuals, which have undergone partial disintegration during depression, are restored to normal morphology are: (1) Growth and regeneration of hypostomes, mouths and tentacles; (2) In specimens which develop two hypostomes, fusion of the hypostomes will occur and extra mouths will be automatically eliminated; (3) In specimens which possess supernumerary tentacles, the number of tentacles is reduced by: (a) absorption of misplaced tentacles by the hypostome, (b) fusion of small or misplaced tentacles with each other and later, absorption by the hypostome, (c) fusion of full sized and normally placed tentacles with each other, (d) changes of positions of tentacles, principally, movement from abnormal positions inside or outside of the regular ring to positions on the ring.

The growth and regeneration of hypostome and tentacles occurs within a day or two after recovery from depression. The fusion of divided hypostomes and the absorption of small and misplaced tentacles requires a few days more and the fusion of normal sized and normally placed supernumerary tentacles may be in progress as long as 80 days after the origin of the supernumerary condition. The fusion of supernumerary tentacles appears to occur step-wise with no overlapping in time of separate tentacle fusions.

The origin of supernumerary tentacles and of divided hypostomes has been observed repeatedly in old individuals recovering from depressions but only once in a bud which was not detached from the parent. In a bud, division of hypostomes usually leads to longitudinal fission.

The fusion of supernumerary hypostomes and tentacles appears to take place independently of budding or the formation of spermaries. However, in the case of long depressions, fusing tentacles may disintegrate if the other tentacles do so, or resume fusion after a depression if the tentacles have not been damaged.

Transverse Fission and Rearrangement of Body Regions

Hyman (1928) observed transverse fission and rearrangement of body regions in four male specimens of *Pelmatohydra oligactis* and their progeny. As a result of heavy feeding or, in some instances, for unaccountable reasons, buds were retained to form temporary colonies after changing their normal positions upon the parents. In some instances, multiple feet were formed in the colonies. Final results indicated that transverse divisions occurred in positions which divided the colonies into equal masses and that single normal individuals were formed eventually with one foot and one hypostome each.

In the present study, four individuals occurred in a male pedigreed stock in which transverse fission was observed. In each case, a bud was retained upon the body of a parent and, after establishing its independence, it functioned as a separate individual. A temporary colonial situation arose often in rapidly budding individuals because a bud would start budding for itself before being separated from the parent. Such conditions were resolved in a matter of a few hours by the separation of the bud from the parent. Instances of bud retention resulting in colony formation, which was resolved by transverse divisions, occurred only in specimens which entered a depression with a bud attached and, on emerging from the depression, retained the bud instead of separating from it. Once the bud had passed the period for separation, it began to reproduce by budding for itself and at the same time the parent was doing likewise. Entirely independent of the process of reproduction in each part of the two-part colony, a regulatory process involving the whole colony was occurring which tended to separate the whole mass into two equal parts, to form new parts where they were required for normal structure and to shift and adjust body regions. All of the four cases observed were somewhat similar and only the most complicated will be described.

Specimen 5.3.3 under maximal feeding had produced 31 buds in 28 days (Diag. 2) and bud number 32 was still attached when the specimen entered a state of depression (Figs. 1 and 2). Recovery from its depression required two days. In Diagram 2, the symbol D–R indicates the period of depression and recovery. Bud number 32 did not separate from the parent on recovery from depression but remained attached and shifted its position nearer to the base of the parent (Fig. 3). Active budding was resumed by the parent and 48 buds were produced in the next 31 days (Diag. 2, I). In the same period of time the retained bud produced 31 buds of its own (Diag. 2, II). Within a few days after resumption of budding by the parent (Fig. 5, I), the budding region began to elongate as indicated by the longer distance between the upper and the lower buds. Two days later, a delicate transverse diaphragm appeared within this elongated budding zone, separating buds in the lower budding zone from the upper budding zone (Fig. 6). The diaphragm marked the beginning of a transverse division and the site in which two new feet were to be formed. In Figure 7, it is indicated clearly that the parent individual (I) had become re-oriented, at least in its budding zone, upon the new foot (N.F.) while individual II had its budding zone oriented upon both the old foot (F.) and the new foot region (N.F.). The differentiation of the new feet, and the separation of the two individuals I and II required 16 days (Diag. 2, T.F.) (Figs. 6–11). It is indicated in Figure 8 that both individuals I and II had become oriented upon the new foot region although the only functional foot for the colony was the old foot (F.). Before the individuals became completely separated and the new feet became functional, the position of the old foot changed progressively (Figs. 8, 9, 10, 11, F.) until the foot became fused with the nearest of the new feet so that in spite of the formation of two new feet in a new position, the actual locus of the attachment of the animal to the substratum remained always the same. The actual separation of individuals I and II had no influence upon the continuity or rate of budding in the two individuals. The termination of the process of transverse fission shown by the right end of the line T.F. in Diagram 2 did not disturb bud production.

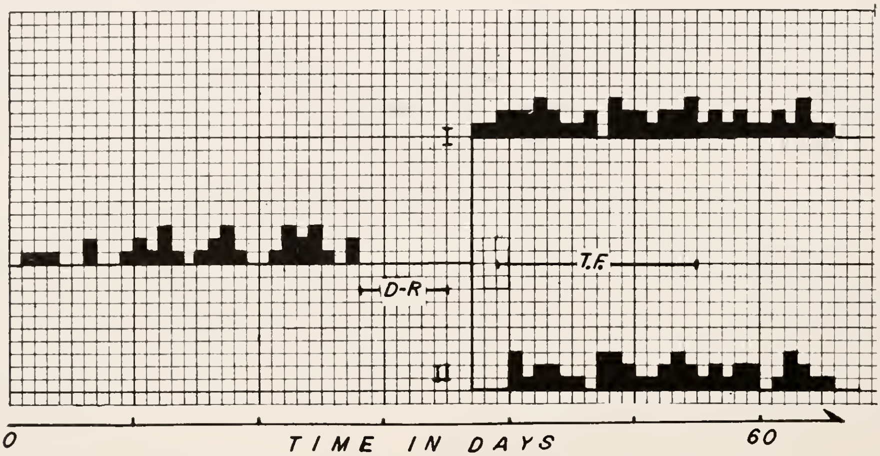

Diagram 2. Block diagram showing relation of reproduction by budding to transverse fission in Specimen 5.3.3. Blocks represent number of new buds produced per day. D-R indicates period of depression and recovery in parent. I represents continuation of that portion of parent above site of transverse division; II represents retained bud and that part of the parent taken over by the retained bud. T.F. represents period from first appearance of transverse diaphragm to the end of fission.

Summary: In the cases observed in this study, transverse fission occurred in temporary colonies produced by retention of a bud by individuals which had just recovered from a depression. In due time, a transverse division occurred in such a position as to divide the colony into two equal masses. New feet arose at the site of the transverse division and re-orientation of body regions, particularly budding zones and new and old feet, occurred so as to produce single normal specimens. No cases of transverse division were observed except in connection with the resolution of temporary colony formation.

Discussion

There is little reason for assuming that transverse and longitudinal fissions are methods of reproduction. It is a fact that fissions result in the formation of additional individuals but it is true also that the fissions assist in resolving abnormalities of form and in restoring normal structure. The opinion of the writer agrees with that of Hyman (1928) (p. 73) "that fission in hydra is not a normal method of asexual reproduction but a mode of regulation of previously existing abnormalities."

The capacity for regulation in *Pelmatohydra* is very extensive and the total capacity is studied better by experiments involving extirpation of parts, coalescence, grafting, etc. Reversals of polarity, regeneration, rearrangements of body parts and other regulatory features may be demonstrated easily. In this paper interest is centered not so much upon regulative capacity as spontaneous regulation in relation to the events of the normal life history of *Pelmatohydra*. Although the types of regulatory processes described by Hyman (1928) and by the writer are rather few it is assumed that other types of spontaneous regulation may occur under appropriate natural conditions.

In some animals (other than hydra) the adults seem to have retained little or no capacity for regulation. In some others regulation appears only spasmodically in the repair of tissues injured by accident or in the correction of structural anomalies arising after accidental injury. Regulation in these cases appears as an extraordinary rather than a normal process. Regulation in *Pelmatohydra* is seen as an almost continuous process because of the frequency of the origin of structural deviations from the clonal type, particularly in tentacle number, and the persistent tendency to correct these deviations even when they have no functional significance. The sequence of events in which regulation plays a part under optimal conditions as found in nature or in the laboratory is roughly as follows: (1) There is a period of reproduction during which buds are produced rapidly, as rapidly as 1.14 buds per day. (2) Each period of rapid reproduction is followed by a period of depression during which control of growth patterns is partially or wholly lost. (3) Growth and regeneration when resumed may give rise to atypical structures. (4) Regulation corrects the atypical character of the structures so that the clonal type of structure is restored. This sequence of events can hardly be considered abnormal for *Pelmatohydra*. Reproduction and depression appear whenever optimal conditions for budding occur. Some phase of atypical form arises frequently and as surely as it arises, regulation follows. In the animals possessing little capacity for regulation, atypical structures would become permanent if they did not interfere with vital processes and they could prevent survival of an animal if they affected important organs. *Pelmatohydra* happens to be a form which has retained great regulative capacity even in the adult and since the formation of atypical structures in the sequence of events indicated above is common, a balancing regulative process is also common.

There has been a tendency in the literature on regulation to emphasize a unitary control for normal morphogenetic processes and regulation. During the embryology of animals with a regulative type of development the forms of embryos can be experimentally altered but on recovery a normal development is re-established. An assumption of unitary control for development both before and after experimental interference with the embryo is logical. However, the assumption of unitary control for both embryonic development and for regulation in *Pelmatohydra* does not lead to clarity. Rather, it seems that the difference between normal morphogenesis and regulation is the conspicuous feature. In embryonic development as well as morphogenesis initiated by budding, the processes are characterized first by cell proliferation, then growth and finally by differentiation. The development is very rapid in buds. The final result is an animal of rather constant form with a specific number of tentacles. In regulation, on the other hand, the changes occur in a fully matured and differentiated animal with atypical structures and the processes which restore the atypical animal to normal form do not occur in embryology. Some of the processes concerned in regulation occur rapidly, *i.e.,* absorption of supernumerary hypostomes and of supernumerary tentacles. Some other processes such as apico-basal division and transverse fission require more time and in one case described above in which there was a persistent reduction of supernumerary tentacles (from 10 to 6) the regulative tendency lasted for 110 days. The existence of a dual set of controls in regulating animals is best seen in those which undergo profuse budding during the regulatory process. In these cases the animal with supernumerary tentacles is giving rise rapidly to numerous buds which bear tentacles in the number characteristic of the clone. At the same time the same animal is slowly reducing the number of its own tentacles by fusion to approximate the number characteristic of the clone. The same end, normal adult structure, is being attained by different processes proceeding in different directions at different rates.

Further evidence for independent control of regulation is seen in the fact that regulation occurs equally well in specimens which are budding profusely or in specimens which are in temporary depression and are not budding. Waves of spermaries arise, reach maximal stages and then wane without affecting the persistent tendency toward regulation in specimens with atypical structure.

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PHYSIOLOGY OF INSECT DIAPAUSE. IV. THE BRAIN AND PROTHORACIC GLANDS AS AN ENDOCRINE SYSTEM IN THE CECROPIA SILKWORM

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In the previous papers of this series (Williams, 1946b, 1947, 1948a) an endocrine basis was described for the production and termination of pupal diapause in the Cecropia silkworm. The onset of diapause was correlated with a temporary failure of the brain in secreting a hormone required for the initiation of adult development. The ultimate release of this "brain hormone" was found to terminate dormancy and to set in motion the process of adult development. The brain was therefore viewed as the organ of primary control over the genesis and termination of diapause in the Cecropia silkworm.

After more detailed examination it became apparent that the brain hormone is fundamentally a tropic factor whose primary target within the diapausing pupa is a second endocrine tissue, the prothoracic glands. The latter, under stimulation of the brain hormone, then secrete the factor reacting with the pupal tissues to terminate diapause (Williams, 1947–1951). The brain and prothoracic glands were therefore considered to function as an endocrine system in controlling the pupal diapause. However, the role of the brain hormone was not fully clarified in the above-mentioned studies. There remained the possibility that the brain hormone might also act directly on the pupal tissues to condition their ultimate response to the prothoracic gland hormone.

The roles of the brain and prothoracic glands have therefore been examined in further detail in the present investigation. Attention has also been focussed on the possibility that, at earlier periods in the life history, the same hormonal system may control the moulting and pupation of the larval insect.

MATERIALS AND METHODS

The present report is based on a study of approximately two thousand Cecropia silkworms (*Platysamia cecropia*). In certain experiments other Lepidoptera, including *Telea polyphemus, Actias luna, Actias selene, Antheraea mylitta, Bombyx mori, Danaus plexippus, Lymantria dispar* and *Prodenia eridania*, were used as donors and recipients of various endocrine organs. The insects were reared in large numbers from eggs obtained from fertile females. In general, the management of the experimental animals was essentially the same as that described previously (Williams, 1946b).

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Carbon dioxide anesthesia was used in all surgical procedures (Williams, 1946a). In experiments performed on pupae, surgical mortality was minimized by implanting several crystals of phenylthiourea and/or a few drops of cyanide Ringer's solution.[2] By temporarily blocking the enzyme tyrosinase, these agents served to protect postoperative pupae from toxic quinone intermediates which appear in the blood if the latter is permitted to darken as a result of the tyrosine-tyrosinase reaction. In certain experiments, a few crystals of streptomycin sulfate and potassium penicillin G were also implanted. These antibiotics virtually eliminated bacterial infection which otherwise complicates certain experiments, especially those performed on isolated pupal abdomens; their use was found to introduce no discernible complications.

The Prothoracic Glands as the Source of the Hormone Terminating Pupal Diapause

In order to induce the adult development of an isolated abdomen it is necessary to implant a brain from a previously chilled pupa, plus prothoracic glands obtained from either chilled or unchilled pupae (Williams, 1947). If the brain functions solely by activating the prothoracic glands, then it should be possible to accomplish this same end in the absence of the brain by implanting prothoracic glands which have already been activated by the brain hormone. Moreover, according to results reported previously (Williams, 1947), the glands should exist in this functional state when obtained from post-diapausing pupae during the first five days of adult development.

Table I

Endocrine activity of prothoracic glands removed at the outset of adult development and tested in brainless pupae or in pupal abdomens

Test preparation	Pairs of glands implanted into each	Number of experiments	Number developing
Pupal Cecropia abdomens	3 from Cecropia	2	1
	3 from Polyphemus	4	2
Brainless diapausing Cecropia pupae	2 from Cecropia	1	0
	2 from Polyphemus	2	0
	3 from Cecropia	2	1
	3 from Polyphemus	3	3
		14	7

Prothoracic glands were therefore removed from animals immediately after the onset of adult development and implanted into isolated pupal abdomens. Experiments of this type were complicated by the fragility and stickiness of the glands which, at the outset of adult development, begin to undergo rapid degeneration;

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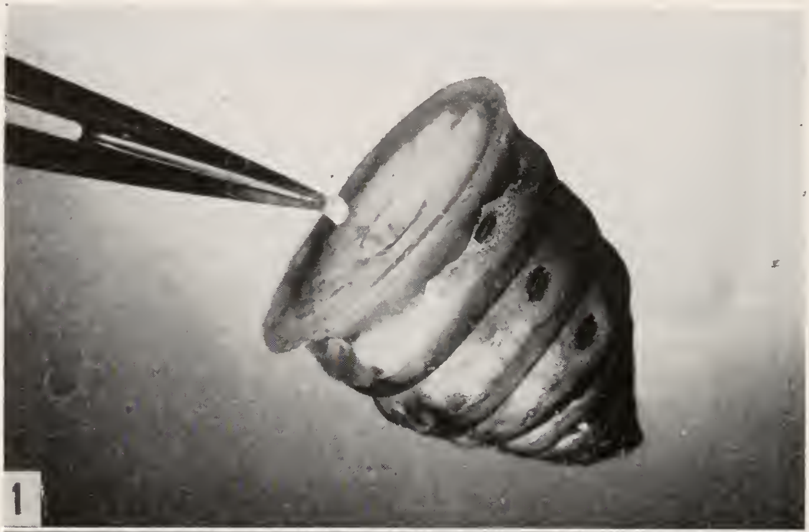
[2] Prepared daily by the addition of 1 part 0.1 *M* potassium cyanide solution to 9 parts of the insect Ringer's solution described by Ephrussi and Beadle (1936). At the pH of the insect, the cyanide exists almost wholly as HCN and within a few days is lost *via* the tracheal system.

ordinarily they could be isolated and implanted only as small fragments. However, by using *Telea polyphemus* as donors, the difficulty was somewhat lessened, since, in this closely related diapausing species, the prothoracic glands are much easier to locate and isolate during the first few days af adult development.

A further serious complication was the high mortality among abdomens receiving such implants. Notwithstanding the use of cyanide and phenylthiourea, the implanted glands caused a delayed darkening of the blood followed by the death



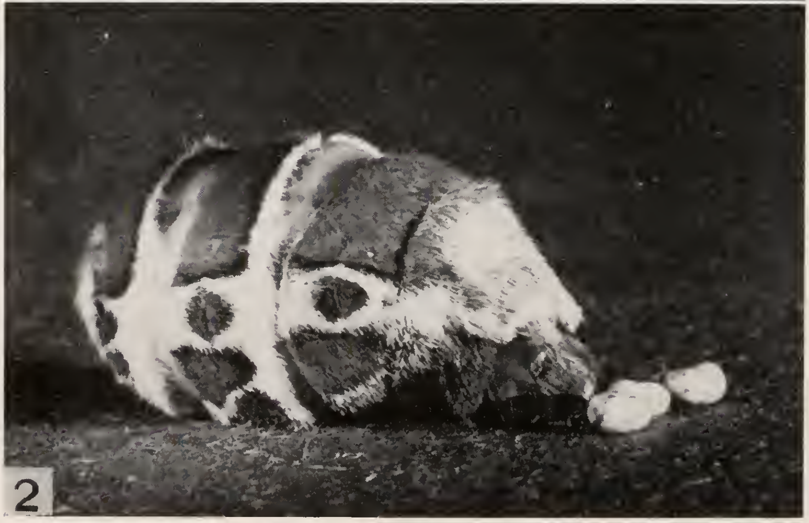


FIGURE 1. Isolated abdomen of a diapausing Cecropia pupa sealed to a plastic slip. Prothoracic glands obtained from postdiapausing pupae are being implanted through a central hole in the slip.

FIGURE 2. The preparation in Figure 1, after adult development. The active prothoracic glands have evoked the metamorphosis of the abdomen; the latter is shown laying several eggs.

of the preparation. This result was not peculiar to the prothoracic glands, but was also commonly observed after implanting fragments of fat-body obtained from developing adults.

Of a total of 25 isolated pupal abdomens, only 6 survived longer than a week following the implantation of prothoracic glands obtained from developing animals (Fig. 1). However, as may be observed in Table I, three of these underwent prompt and complete adult development (Fig. 2).

A lower mortality was encountered in experiments testing the glands in brainless diapausing pupae rather than in isolated abdomens. Of a total of 8 viable preparations of this type, four underwent adult development (Table I).

In view of the injury and fragmentation of the glands during the experimental procedure, the high proportion of negative results is understandable. However, the fact that adult development occurred in 7 of the total of 14 viable preparations is, in itself, highly significant. It demonstrates that the pupal tissues do not require prior reaction with the brain hormone in order to respond to the prothoracic gland hormone.

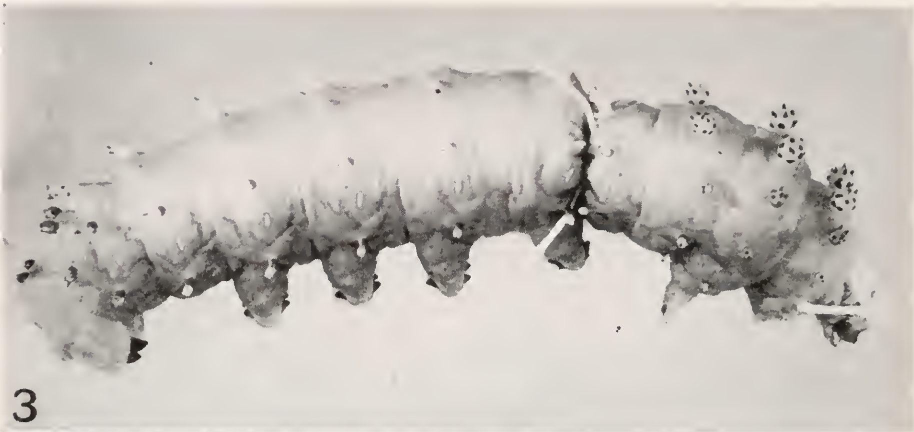
LIGATION EXPERIMENTS ON MATURE LARVAE

Efforts were made to determine whether the brain and prothoracic glands control pupation as well as adult development. In view of the difficulties inherent in surgical procedures on caterpillars, the technique of ligation was applied to mature fifth instar Cecropia silkworms.

As illustrated in Figure 3, two transverse ligatures were placed around each larva, one between the head and prothorax and the other between the second and third abdominal segments. In this manner each animal was subdivided into three blood-tight compartments—a cephalic compartment containing the brain, corpora cardiaca, and corpora allata; a primarily thoracic compartment containing the prothoracic glands; and an abdominal compartment containing no recognized endocrine glands.

The isolated head promptly died, but, as indicated in Table II, the behavior of the thoracic and abdominal compartments was determined by the stage of maturity of the silkworm at the time of ligation. Larvae subdivided just prior to the initiation of spinning underwent no further development, although both thoracic and abdominal compartments continued to live for about a month thereafter (Fig. 3). When the ligatures were applied during the first day of spinning, the abdomen remained larval, whereas the thorax either remained larval or pupated during the following two weeks. Silkworms subdivided during the second day of spinning retained larval abdomens, but all of the thoracic compartments underwent pupation about two weeks thereafter (Fig. 4).

The pupation of the larval abdomen became possible only when the ligatures were applied after the initiation of the prepupal stage. At 25° C. this stage begins four to five days after the initiation of spinning and is accompanied by the eversion of the wing disks and the retraction of the hypodermis from the larval cuticle. In the intact animal this retraction may be recognized by the withdrawal of the pigment granules underlying the transparent larval ocelli (Kühn and Piepho, 1936). Silkworms ligated at the first sign of pigment retraction retained larval abdomens



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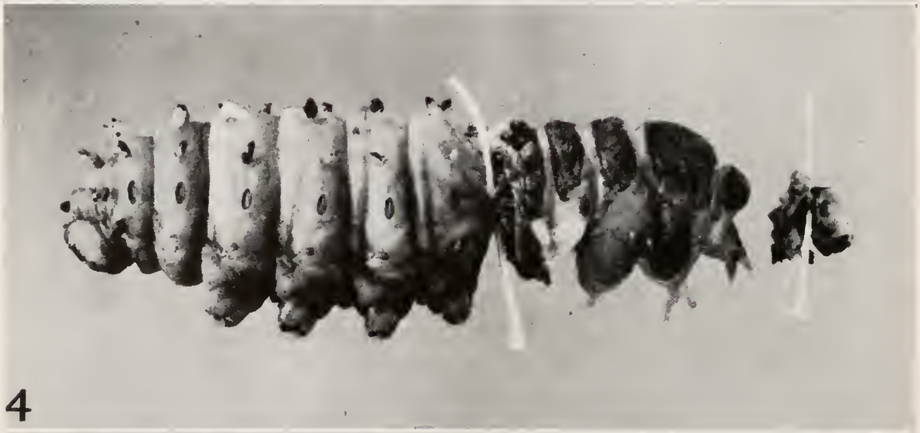

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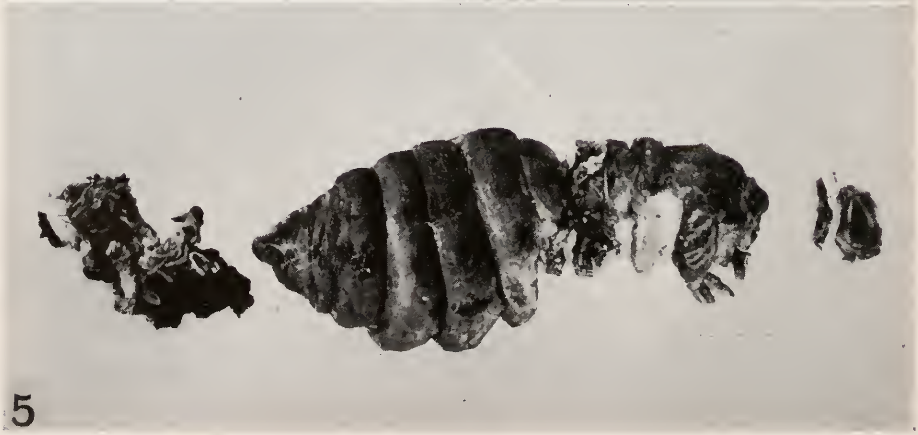

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FIGURE 3. A mature Cecropia larva ligatured behind head and thorax prior to the initiation of spinning. Neither end is able to metamorphose.

FIGURE 4. The same as Figure 3, except that the ligatures were applied during the second day of spinning. The thorax has pupated, but the abdomen remains larval.

FIGURE 5. The same as Figure 3, except that the ligatures were applied after the initiation of the prepupal stage. Both the thorax and abdomen have pupated. The dead larval head and moulted larval cuticle are also illustrated.

in 17 of the 21 preparations. However, in animals ligated a day later, pupation of the abdominal compartment was consistently observed (Fig. 5).

These simple experiments demonstrate that pupation is under the control of both the head and the thorax. The head makes its contribution prior to a critical period signalled by the completion of the outer capsule of the cocoon and, thus, 9 days before the pupal moult. Pupation then comes under the control of the thorax until a critical period signalled by the onset of the prepupal stage.

TABLE II

Behavior of thoracic and abdominal compartments of double-ligatured Cecropia silkworms

Days at 25° C.	Corresponding stage	Number of experiments	Final state of	
			Thorax	Abdomen
0	Just prior to spinning	5	Larval	Larval
0 to 1*	Spinning outer capsule	14	Larval or pupal	Larval
1 to 2	Spinning inner capsule	18	Pupal	Larval
2	Just finished spinning	17	Pupal	Larval
4 to 5**	Earliest retraction of ocellar pigment	21	Pupal	Larval or pupal
5	Full retraction of ocellar pigment	4	Pupal	Pupal
5 to 9	Prepupa	4	Pupal	Pupal
9 to 10	Pupal moult	—	Pupal	Pupal

* "Critical period" for brain.
** "Critical period" for prothoracic glands.

Those thoracic or abdominal compartments which pupated underwent no further development though they continued to live for up to three months thereafter. This behavior is understandable since, as we have seen, the pupal-adult transformation once again requires the hormonal participation of a cephalic organ, the brain, and a thoracic organ, the prothoracic glands.

ABILITY OF LARVAL BRAINS TO TERMINATE PUPAL DIAPAUSE

Efforts were made to determine whether the larval brain controls pupation *via* the same brain hormone, which, at a later stage in the life history, controls adult development. To this end, brains were dissected from caterpillars at precise stages, freed from frontal ganglia and other attached organs, and implanted into the tips of the abdomens of brainless diapausing pupae. Two brains were implanted into each preparation.

The results recorded in Table III demonstrate the ability of the larval brain to substitute for the pupal brain in terminating diapause. In one or more experiments, the brains were found active at every stage in larval and prepupal life.

However, it will also be observed in Table III that many of the brain implants failed to promote adult development and, even when they did so, considerable variation was encountered in the time required for the initiation of development.

When observed through an overlying plastic window, it became apparent that the implanted brains attained intimate connections with the tissues of the host prior to exerting their effect. Minute nerve fibers grew out from the brain and tracheal connections grew in from the surrounding tissues of the host. The subsequent history of such implants was therefore largely uncontrollable—a fact which was probably responsible for a large fraction of the observed variability.

TABLE III

Endocrine activity of larval brains implanted into brainless diapausing Cecropia pupae (two brains into each)

Stage of donors	Number of experiments	Number developing	Days for initiation of development* at 25° C.
Mature 1st instar	2	1	50
1st moulting to 2d	3	1	195
Early 2d	2	2	20; 251
Mature 2d	3	1	17
2d moulting to 3d	2	2	13; 226
Early 3d	1	1	228
3d	2	2	10; 181
Mature 3d	3	2	21; 139
3d moulting to 4th	2	2	25; 217
Early 4th	2	2	28; 110
4th	2	2	48; 257
Mature 4th	4	3	16; 51; 173
4th moulting to 5th	4	3	65; 177
Early 5th	17	12	12; 18; 21; 23; 25; 47; 48; 58; 73; 112; 225;—
Mature 5th	18	9	11; 48; 107; 137; 143; 180; 218; 221; —
Spinning outer capsule	19	8	12; 17; 18; 28; 39; 55; 112; 190
Spinning inner capsule	13	6	65; 71; 200; 210; 240; 520
Finished spinning	11	5	65; 129; 134; 152; 166
Early retraction of ocellar pigment	6	5	80; 97; 130; 153; 187
Prepupa	7	5	64; 140; 159; 165; 225
Fresh pupa	10	0	—

* Positive experiments only.

Consequently, on the basis of the present data, it is impossible to judge whether any systematic change occurs in the brain's endocrine activity between the first larval stage and the initiation of spinning. However, it is clear that during the first day of spinning the brain is as active as at any stage in larval life. Yet, within the nine days that follow, a rapid decline takes place in its endocrine activity. By the time of the pupal moult, the brain is totally inactive when tested. The net result is that the newly formed pupa is equipped with a brain which is incompetent to secrete the brain hormone.

ABILITY OF LARVAL PROTHORACIC GLANDS TO TERMINATE PUPAL DIAPAUSE

According to the preceding analysis, the larval brain secretes the same brain hormone in promoting pupation as does the pupal brain in promoting adult development. An extension of this principle to the prothoracic glands suggests that the same prothoracic gland hormone controls both pupation and adult development. This hypothesis was tested in brainless diapausing pupae by implanting prothoracic glands obtained from caterpillars at various stages during late larval and prepupal life. Two pairs of glands were implanted into each pupa.

As recorded in Table IV, larval glands removed prior to the initiation of spinning caused adult development to occur in only two of the 23 preparations, whereas glands removed after the initiation of spinning and prior to the prepupal period were active in 7 of 11 preparations. Glands removed after the onset of the prepupal stage were inactive.

TABLE IV

Endocrine activity of larval prothoracic glands implanted into brainless diapausing cecropia pupae (two pairs into each)

Stage of donors	Number of experiments	Number developing	Days for initiation of development* at 25° C.
Mature 4th instar	4	0	—
4th moulting to 5th	2	0	—
Early 5th	9	1	27
Mature 5th	8	1	19
Spinning outer capsule	2	2	16; 21
Spinning inner capsule	5	2	15; 16
Finished spinning	3	3	23; 240; 480
Early retraction of ocellar pigment	3	0	—
Prepupa	1	0	—
Pupa	5	0	—

* Positive experiments only.

From these experiments we learn that the larval prothoracic glands can be made to substitute for the pupal glands in terminating pupal diapause. Since the glands were tested in brainless pupae, the experiments also demonstrate that the larval glands usually exist in the activated state during a five-day period beginning with the initiation of spinning and ending with the onset of the prepupal stage.

PUPATION INDUCED BY PUPAL ENDOCRINE ORGANS

In the experiments just considered, the larval brain and prothoracic glands were able to provide the necessary hormonal stimulus for the adult development of the pupa. Can the corresponding pupal organs evoke the pupation of the larva?

A preliminary series of experiments indicates an affirmative answer to this question. In three of a total of six preparations, "permanent" larval abdomens, isolated by ligation prior to spinning, were induced to pupate by implanting brains and prothoracic glands obtained from chilled pupae.

PARABIOSIS EXPERIMENTS

In our progress up to this point, support has been found for the view that the prothoracic glands secrete the metamorphosis hormone under tropic stimulation of the brain hormone. Further insight into the function of this endocrine system is afforded by experiments where brainless pupae were joined in serial parabiosis.

A series of Cecropia pupae were first stabilized in "permanent" diapause by removing their brains and replacing the pupal cuticle and hypodermis at the site of the operation by a transparent plastic facial window (Williams, 1946b, 1947). In like manner, plastic windows were established in each pupa at the thoracic tergum and the tip of the abdomen. A week later, a window was removed from each individual and the pupae united in pairs, the thorax of the one being sealed with melted paraffin to the tip of the abdomen of the other. Ten days later the pairs of pupae were united by a continuation of the above-mentioned procedure. In this manner, chains of four to ten brainless pupae were established in serial parabiosis.[3] In approximately a week the operative sites underwent a process of repair accompanied by a growing together of the hypodermis of successive individuals, yielding, as it were, a single elongate organism possessing continuity of blood and hypodermis. Though each individual retained prothoracic glands within its thorax, none possessed a brain within its head. For this reason the diapause was persistent and three chains of pupae died several months later without any indication of adult development.

Experiments were performed to ascertain the effects of implanting a brain into such preparations. Accordingly, a single brain from a previously chilled pupa was implanted under the facial window of the first animal in each chain, its effects being noted by daily observations through the transparent facial windows. The five viable preparations of this type yielded essentially similar results. The behavior of the preparation illustrated in Figures 6 and 7 may be summarized as follows:

0 day—brain implanted into #1.
17th day—#1 and #2 show initiation of development; no development of #3 to #8.
20th day—#3 and #4 show initiation of development; no development of #5 to #8.
21st day—#5 shows initiation of development; no development of #6 to #8.
30th day—#6 and #7 show initiation of development; no development of #8.
32nd day—#8 shows initiation of development.
38th day—#1 and #2 have completed adult development.
41st day—#3, #4, and #5 have completed adult development.
50th day—#6 and #7 have completed adult development.
52nd day—#8 has completed adult development.

Thus we observe that the single brain set in motion a process of activation which terminated the diapause of each animal in turn. This process began at the anterior

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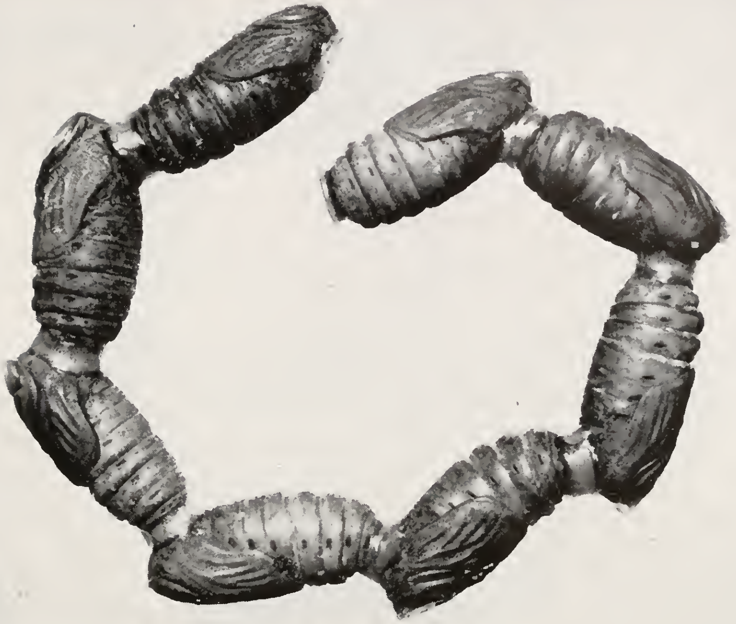
[3] The principal difficulty in establishing these chains is that an infection or darkening of the blood in any one animal promptly spreads throughout the chain and causes the death of all individuals. It is therefore advisable to subdivide the total procedure into several stages spaced at least a week apart. In this manner one may confirm the viability of each animal before proceeding to the next step in assembling the chain.



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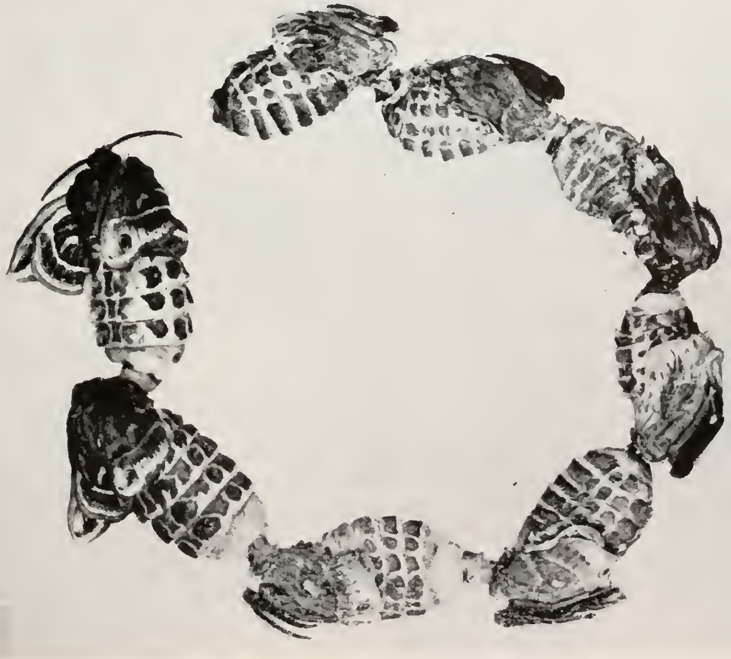

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FIGURE 6. A chain of eight brainless diapausing pupae has been established and a single chilled pupal brain implanted under the facial window of the anterior-most individual. Approximately life size.

FIGURE 7. The preparation in Figure 6 after seven and one-half weeks. The single brain has caused all the pupae to undergo adult development.

end of the chain and required 15 days to travel 24 cm. to the tip of the eighth individual. The chain of pupae yielded a chain of moths, complete both externally and internally. Successive individuals were inter-connected at the site of parabiosis by a hollow bridge of integument.

Light is shed on the role of the brain in such preparations by the experiment illustrated in Figures 8 and 9. Here a chain of 6 brainless diapausing pupae was established and a brain implanted into #1. When #1 and #2 showed the earliest initiation of development, #1 containing the brain, and #6 were detached. As illustrated in Figure 9, the activation, now in the absence of the brain, continued beyond #2 and traversed the residual three pupae in the chain. All the animals completed adult development save #6; the latter died 18 months later without any indication of development.

The failure of the hindmost animal to develop after detachment indicates that a threshold titer of the brain hormone had not been distributed throughout the chain prior to the initiation of development of animals #1 and #2. Yet the detachment of the brain-containing pupa did not interfere with the further non-decremental spread of activation from animal #2 to #3 and from #3 to #4 and from #4 to #5. It seems necessary to conclude that the brain hormone acted locally at the anterior end of the chain to initiate a reaction which, without further dependency on the brain or the brain hormone, could be duplicated in each successive pupa.

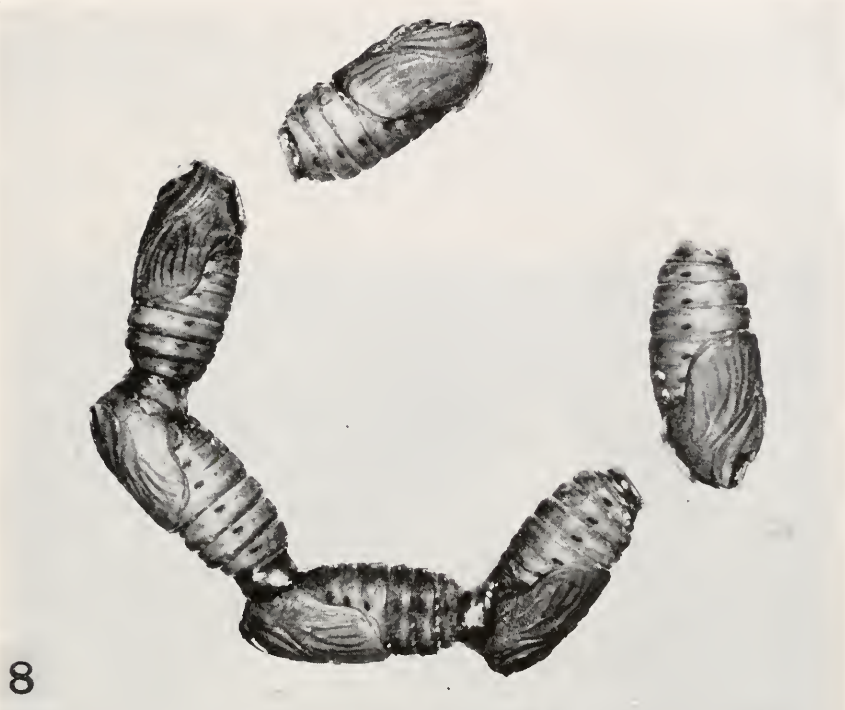
The fact that each pupa contained prothoracic glands appeared to afford a rational basis for such a self-sustaining reaction. According to this interpretation, the brain hormone triggered the prothoracic glands of animal #1 and the latter's hormone then spread to animal #2 and triggered its prothoracic glands. A continuation of this process would lead to the recruitment of prothoracic gland activity in each successive pupa—the glands being triggered by the prothoracic gland hormone itself arising in the preceding pupa.

Under this point of view, the self-sustaining character of the process should not appear if prothoracic glands were present only in the anterior-most insect. This prospect was tested experimentally.

Since it is technically impossible to extirpate the prothoracic glands of Cecropia, the experiment was performed on chains of isolated abdomens. Twenty chilled pupae were transected just behind the prothoracic glands; *i.e.,* at the meso-metathoracic level. Crystals of penicillin and streptomycin were implanted at this time to minimize the chance of infection. Each abdomen was then sealed with melted paraffin to a plastic slip containing a central hole 5 mm. in diameter; the latter was temporarily plugged with melted paraffin. Each abdomen was further provided with a plastic window at the tip of the abdomen. Twelve of the 20 abdomens survived this treatment and were in good condition a week later. The paraffin plugs and the abdominal windows were then removed and pairs of pupae

FIGURE 8. A chain of six brainless diapausing pupae has been established and a single chilled pupal brain implanted under the facial window of the anterior-most individual. When pupae 1 and 2 showed the initiation of development, pupae 1 and 6 were detached. Approximately life size.

FIGURE 9. The preparation in Figure 8 seven weeks later. After detachment of the brain-containing pupa, the activation has continued to spread down the chain of interconnected pupae. The hindmost pupa fails to develop after detachment. Approximately life size.



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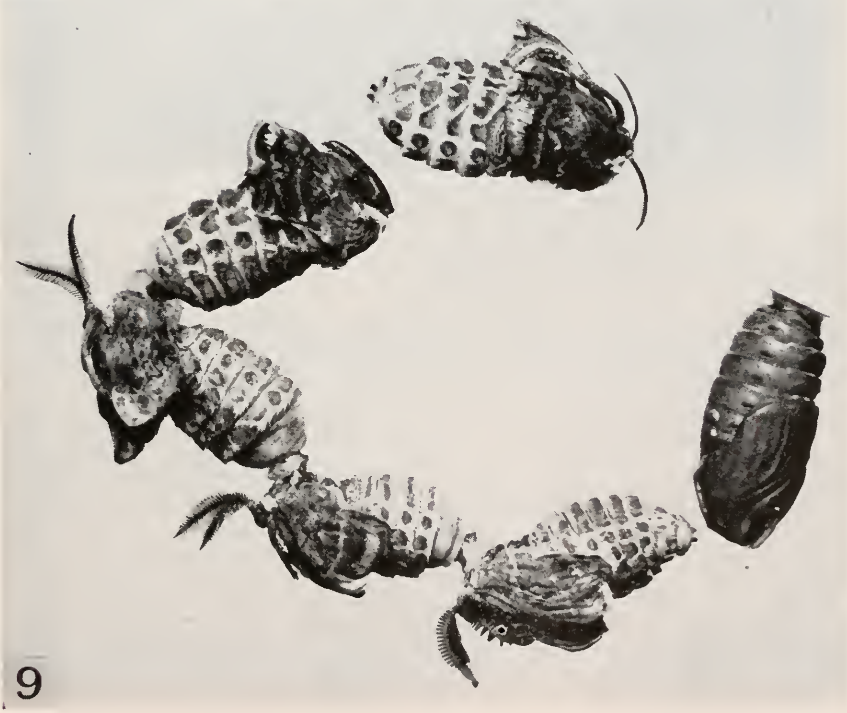

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established and sealed together with melted paraffin. Ten days later, the pairs were joined in sequence by a continuation of this procedure. Finally a normal chilled pupa was grafted to the anterior end of each chain of four abdomens (Fig. 10). Two such chains were prepared, plus one control preparation in which a chilled pupa was joined to a single abdomen.

In the case of the control preparation, the pupa initiated adult development on the 12th day and the attached abdomen on the 14th day. Both showed complete adult development on the 35th day.

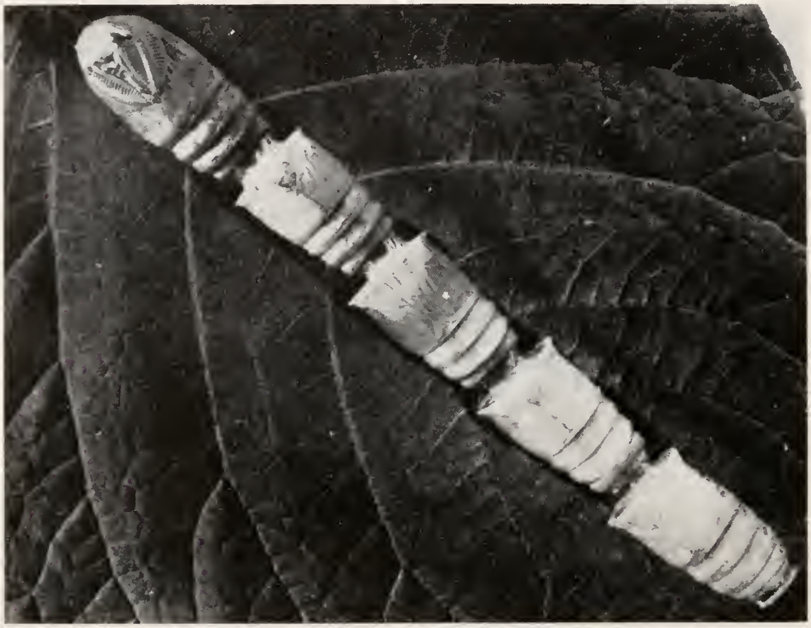

FIGURE 10. A chilled pupa, containing both a brain and prothoracic glands, has been attached to a chain of four pupal abdomens lacking these endocrine organs. The activation here spreads decrementally: the anterior-most animals undergo adult development, the hindmost animals do not. Approximately life size.

One of the two experimental preparations survived. The pupa initiated development on the 14th day, abdomen #1 on the 15th day, and abdomen #2 on the 18th day. Abdomens #3 and #4 showed no development.

On the 38th day the pupa and abdomen #1 showed complete adult development. Abdomen #2 showed only the earliest stage of development, while abdomens #3 and #4 showed no development. The connections between successive abdomens remained patent and, by pressing on the abdomens, the blood could be propelled between the various members of the chain. The moth and the adult abdomen #1 were at this time detached from the chain. Though the latter survived until the 80th day, none of its members underwent further development.

The behavior of the chain of abdomens therefore stands in marked contrast to that observed in the previous series of experiments where each member of the chain possessed prothoracic glands. When each member possessed prothoracic glands, then the activation spread without decrement and could apparently cause the development of any number of brainless pupae that one incorporated into the chain. But when only the anterior-most animal possessed prothoracic glands, then the activation spread decrementally and only the next adjacent member of the chain received the necessary concentration of prothoracic gland hormone.

Just such a difference in the behavior of the two types of preparations would be anticipated if the prothoracic glands of successive individuals can be triggered by the prothoracic gland hormone itself, arising in the preceding member of the chain. Manifestly, within the normal insect the operation of this mechanism would assist the integration of the endocrine mechanism. Since the prothoracic glands are paired organs, whose respective thresholds to brain hormone may differ, the sensitivity of one prothoracic gland to the other's hormone would serve to synchronize their secretory activities and couple the two glands into a functional unity.

Discussion

The experimental results summarized in Figure 11 demonstrate that both pupation and adult development are controlled by an endocrine system consisting of the brain and prothoracic glands. According to the ligation experiments (Table II), pupation requires an initial stimulus from the larval brain and a subsequent stimulus from the larval prothoracic glands. As diagrammed in Figure 11A, the brain completes its contribution to pupation during the first day of spinning, *i.e.*, 9 days prior to the pupal moult, and then declines in endocrine activity (Table III).

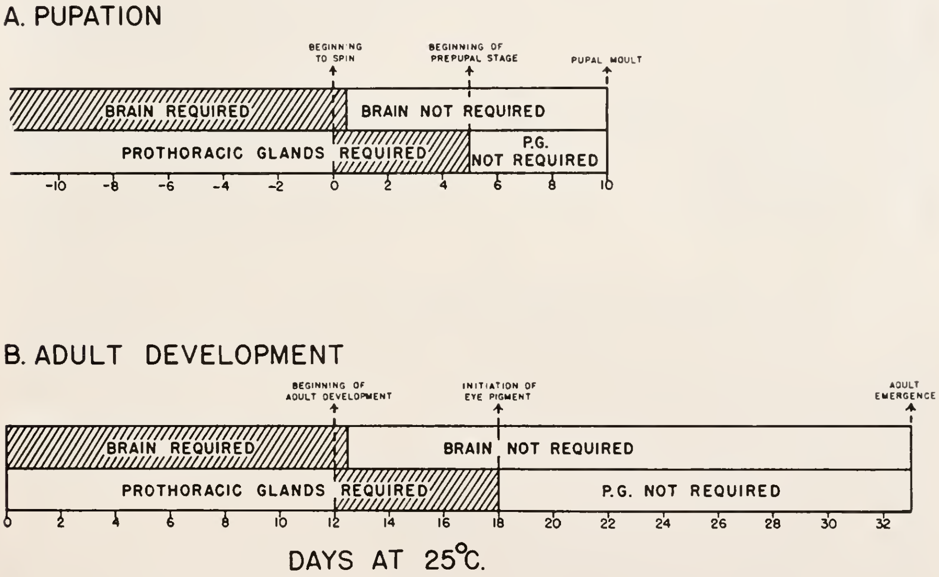
A. PUPATION
BEGINNING TO SPIN
BEGINNING OF PREPUPAL STAGE
PUPAL MOULT
BRAIN REQUIRED
BRAIN NOT REQUIRED
PROTHORACIC GLANDS REQUIRED
P.G. NOT REQUIRED
-10
-8
-6
-4
-2
0
2
4
6
8
10
B. ADULT DEVELOPMENT
BEGINNING OF ADULT DEVELOPMENT
INITIATION OF EYE PIGMENT
ADULT EMERGENCE
BRAIN REQUIRED
BRAIN NOT REQUIRED
PROTHORACIC GLANDS REQUIRED
P.G. NOT REQUIRED
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
DAYS AT 25°C.

FIGURE 11. The endocrine control of the pupation of the larva (A) and the adult development of the previously chilled pupa (B) at 25° C. For explanation see text. The cross-hatching records the periods when the brain or prothoracic glands were found maximally active.

As also illustrated in Figure 11A, the termination of brain function ushers in a five-day period of prothoracic gland function, beginning with the onset of spinning and ending with the onset of the prepupal stage. The further development of the pupa then becomes independent of the prothoracic glands. The latter remain intact, but show a rapid loss of endocrine activity (Table IV).

Within the pupa the function of the prothoracic glands in promoting adult development once again requires the tropic stimulus of the brain hormone. But in the Cecropia silkworm, as we have seen, this stimulus is not forthcoming for several months after pupation, until the diapausing brain has recovered its endocrine competency under the influence of low environmental temperature.

When such a chilled pupa is placed at 25° C., the events which terminate its diapause are strikingly similar to those which controlled its pupation. As diagrammed in Figure 11B, the secretion of the brain hormone occurs promptly at the high temperature, proceeding at a rate determined largely by the duration of the brain's prior exposure to low temperature (Williams, 1952). The brain hormone attains threshold titer after a specific period, diagrammed as 12 days in Figure 11B. Then, within a few hours, a remarkable series of events takes place: (1) the prothoracic glands, which have been inactive since the prepupal stage, are triggered by the brain hormone; (2) the prothoracic gland hormone reacts with the pupal tissues to evoke the initiation of adult development (Table I); and (3) the brain becomes dispensable and undergoes a rapid decline in endocrine activity (Williams, 1947).

For six days following the termination of diapause, the further progress of adult development continues to require the secretory activity of the prothoracic glands. But, unlike their behavior prior to pupation, the glands undergo complete degeneration during this first week of adult development. Notwithstanding this fact, the prothoracic gland hormone apparently persists within the animal until adult emergence and is thus in a position to influence the later stages of adult development (Schmidt and Williams, 1949).

According to the preceding analysis, the pupal diapause is terminated by the recurrent function of the same endocrine system that controls pupation itself. Moreover, the demonstrated ability of larval brains and prothoracic glands to substitute for the corresponding pupal organs indicates that the same brain hormone and prothoracic gland hormone control both pupation and adult development.

In relation to both events we observe that the termination of brain function is synchronized with the initiation of a prothoracic gland function—a sequence which argues that the brain hormone is solely a tropic stimulus for the prothoracic glands. This inference is greatly strengthened by the finding that larval or pupal prothoracic glands, previously exposed to the brain hormone, were able to evoke adult development in the absence of the brain (Table I). Though the brain gives leadership to the endocrine events which preside over pupation and adult development, it apparently does so *via* the tropic action of its hormone on the prothoracic glands.

In agreement with Fukuda's investigation of *Bombyx mori* (1944), the present findings therefore serve to emphasize the significance of the prothoracic gland hormone, since it is apparently this factor which reacts with the larval tissues to induce pupation and with the pupal tissues to induce adult development.

Attempts to determine whether the brain and prothoracic glands also participate in the hormonal control of larval growth and moulting are complicated by technical difficulties. For as Bounhiol (1938) has emphasized, the moulting or pupation of most insects becomes possible only when the animal attains a certain nutritional status. Each larval instar therefore begins with a period of *alimentation indispensable,* followed by a period of *alimentation facultative.* During the first of these periods metamorphosis is blocked by any treatment which prevents further feeding—such measures, for example, as ligation, brain removal, or simple starvation. In consequence, a direct approach to the analysis of the hormonal control of moulting has proved feasible only in *Rhodnius* where, at the beginning of each instar, the period of *alimentation indispensable* is limited to a single blood meal (Wigglesworth, 1940, 1951). Studies of this type have proved difficult or impossible in most other insects where the hormonal control of moulting is usually exercised during the period of *alimentation indispensable.*

Indeed, our understanding of the hormonal control of pupation is obscured by this difficulty in certain species. Unfortunately, this appears to be so in a favorite experimental animal, *Bombyx mori.* Here the brain apparently secretes a threshold titer of its hormone early in the final larval instar when most experimental procedures are impossible. This fact was adequately appreciated by Bounhiol (1938) and probably accounts for Fukuda's (1944) failure to demonstrate a role of the brain in the pupation of *B. mori.*

Notwithstanding these complications, there is circumstantial evidence that the brain and prothoracic glands also participate in the regulation of larval growth and moulting when, in the immature insect, they function in conjunction with the corpora allata's "juvenile" or "status quo" hormone. This conclusion is supported by two lines of evidence: (1) the demonstrated ability of Cecropia brains to secrete the brain hormone as early as the first larval instar; and (2) Fukuda's studies (1944) of *B. mori* where the moulting of the immature insect appeared to require the function of a prothoracic center.

Thus, at all stages in post-embryonic development, the picture which gradually takes shape finds the prothoracic glands supplying an apparently direct stimulus for cellular growth and differentiation. Functioning in the presence of the corpora allata's "juvenile" or "status quo" hormone, the prothoracic gland hormone promotes the growth and moulting of the immature larva; functioning in the absence of this conservative factor, it promotes the rapid strides in growth and differentiation which culminate in the pupation of the mature larva and the adult development of the pupa.

This conclusion based on studies of the Cecropia silkworm and its relatives is in substantial agreement with Fukuda's (1944) results on *B. mori* and will probably apply to all Lepidoptera. Indeed, recent studies of the Diptera (Possompes, 1949, 1950a, 1950b), the Blattoidea (Bodenstein, 1951), and Hemiptera (Wigglesworth, 1951) suggest that, in all metamorphosing insects, the prothoracic glands and their homologues are the source of the factor which has variously been termed the "moulting," "pupation," or "growth and differentiation" hormone.

Consequently, the control which the prothoracic gland hormone exercises over pupation and adult development in the Cecropia silkworm is, most probably, the expression of a generalized phenomenon. The pupal diapause, under this point of view, finds an endocrine basis in the latent operation of a normal mechanism shared with non-diapausing species—the secretion of the prothoracic gland hormone. And according to the results of the present investigation, this latency, in turn, results from a loss of the endocrine activity of both the brain and the prothoracic glands during the prepupal period. Adult development becomes possible only when the brain recovers its secretory powers and repeats the tropic stimulation which it had administered to the prothoracic glands prior to the prepupal period.

In the case of non-diapausing species, where the prothoracic glands promote prompt adult development by secreting their hormone a few days following pupation, the timing of the endocrine events appears to be modified in one of three directions: (1) both the brain and prothoracic glands may remain active following pupation; or (2) the brain may remain active within the newly found pupa while the prothoracic glands become temporarily inactive; or (3) the brain may become inactive following pupation while the prothoracic glands remain active. All of these circumstances are consistent with the function or activation of the prothoracic glands and, therefore, with the prompt onset of adult development; all will probably be encountered when a sufficiently large number of non-diapausing species is examined.

In the case of species incapable of pupal diapause, the first or third adaptations appear to be the rule. Thus the adult development of *Bombyx* (Bounhiol, 1938; Fukuda, 1944), *Deilephila* (Caspari and Plagge, 1935; Plagge, 1938), *Galleria* (Bounhiol, 1938), *Lymantria* (Kopeć, 1922; Bounhiol, 1938), *Phryganidia* (Bodenstein, 1938), and *Vanessa* (Hachlow, 1931) is known to proceed normally in brainless pupae. Our studies show this to be true also in the non-diapausing pupae of the monarch butterfly, *Danaus plexippus*, and the southern armyworm, *Prodenia eridania*.

In the case of the bivoltine silkworm, *Actias luna*, and the polyvoltine *Actias selene*, the initial brood of non-diapausing pupae undergoes prompt adult development *via* the second adaptation mentioned above. Within the newly formed pupa the brain is active and the prothoracic glands inactive. The pupa is therefore converted to diapause if the brain is removed immediately after pupation. However, the subsequent diapausing brood of *Actias luna* shows the same timing of events as observed in Cecropia in that the brain as well as the prothoracic glands are inactive within the newly formed pupa.

Thus, in our understanding of the endocrinology of metamorphosis, we simultaneously perceive a simplification and an increasing complexity—simplification in the operation of a potent growth factor, the prothoracic gland hormone, in controlling growth and differentiation at all stages in post-embryonic development; increasing complexity in that secretion of this hormone is under the tropic control of the brain.

The photographs in Plate I, II, and V were made in collaboration with Dr. Roman Vishniac and are used with the permission of *Time,* Inc. Mr. Dietrich Bodenstein, Dr. Leigh E. Chadwick, and Dr. Berta Scharrer were most helpful in reading the paper in manuscript form; the presentation has profited greatly by their criticisms and suggestions.

SUMMARY

1. The pupation and adult development of the Cecropia silkworm are under the control of a hormonal system consisting of the brain and prothoracic glands. The adult development of the pupa is controlled by the same "brain hormone" and "prothoracic gland hormone" which, at an earlier stage in the life history, control the pupation of the larva.

2. Though the brain gives leadership to the endocrine events that preside over pupation and adult development, it apparently does so by supplying a tropic hormonal stimulus for the prothoracic glands. The latter's "growth and differentiation hormone," secreted under stimulation of the brain hormone, then reacts with the larval tissues to promote pupation or with the pupal tissues to promote adult development.

3. The pupal diapause is a state of endocrine deficiency resulting from a temporary failure of the brain in secreting its tropic hormone following pupation. What the pupal tissues require is the prothoracic glands' growth and differentiation hormone. But this factor becomes available only after the brain recovers its secretory ability and triggers the function of the prothoracic glands.

4. Evidence is presented that the prothoracic glands can be triggered also by the prothoracic gland hormone itself. This device, it is suggested, serves to couple the paired glands into a functional unity and assure their simultaneous response when the brain hormone is released.

5. Evidence of a more circumstantial character indicates that larval growth and moulting are also promoted by the prothoracic glands' growth and differentiation hormone, acting within the immature insect in conjunction with a conservative factor secreted by the corpora allata. The corpus allatum hormone plays no positive role in the pupation or adult development of the Cecropia silkworm.

6. The endocrine system of Cecropia is compared to that of bivoltine and non-diapausing Lepidoptera. In Cecropia, both the brain and prothoracic glands become inactive after providing the endocrine stimulus for pupation. Prompt, non-diapausing development becomes possible in those species where either the brain or the prothoracic glands retain their endocrine activity within the newly formed pupa.

7. The pupal diapause therefore results from the delayed function of a normal endocrine mechanism shared with non-diapausing species.

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THE PROTOPLASMIC CORTEX IN RELATION TO STIMULATION[1]

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In spite of the fact that the protoplasmic cortex is undoubtedly a most important part of the cell, little is known concerning its physical properties and how these may change during the life and activity of the cell. Typically, living cells consist of a mass of fluid protoplasm surrounded by a stiff layer of protoplasm just underneath the cell membrane. This stiff layer constitutes the cortex. A recognizable cortex has been described for ameba and for various types of marine egg cells. Such a cortex also exists in striated muscle cells (Rieser, 1949), and there is a likelihood that all cells have a cortex. In a marine egg cell the organization of the embryo and the course of development may well depend on the physical arrangement of the cortex, for this is often the only part of the egg protoplasm that is firm, and it is difficult to conceive of organization in a highly fluid mass of material. In irritable cells, the cortex may be the sensitive portion of the living structure. This would be a logical enough assumption, for the outer portion of the protoplasm presumably would be the first to become affected by external stimuli.

The colloidal theory of stimulation and response states that typically the first action of a stimulating agent is on the cortex of the cell. The stimulus causes a release of bound calcium from the cortex and this calcium passing into the cell interior causes changes in the protoplasm there. The evidence for this theory is many-sided and need not be discussed here (see Heilbrunn, 1951, for a recent summary). In the ameba, it is certain that various types of stimulating agents do cause a liquefaction of the cortex, and this liquefaction is thought to be associated with a release of calcium ion. But a weakness of the colloidal theory lies in the fact that it is only in the ameba that such changes in the cortex have actually been observed. This is due to the fact that although various types of cells are known to have a cortex, this cortex is ordinarily so stiff that it can not readily be studied.

Fortunately the egg of the worm *Chaetopterus pergamentaceus* has a cortex with easily visible granules and these can be moved by centrifugal forces not violent enough to break the cell. By measuring the amount of centrifugal force necessary to move granules out of the cortex, it is possible to estimate the stiffness of the cortical gel under one condition or another (compare Heilbrunn, Wilson and Harding, 1951, and especially Wilson, 1951).

We were interested in finding out first whether the cortex of the Chaetopterus egg cell was similar in its properties to the cortex of the ameba. The ameba cortex is liquefied by agents which tend to remove calcium from it (oxalates or potassium salts) ; it becomes stiffer in the presence of excess calcium. Likewise both heat and

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cold cause a liquefaction of the ameba cortex; ultraviolet radiation has a similar effect, and so also do fat solvents. We wanted to know therefore whether or not the Chaetopterus egg cortex behaves in this manner.

Secondly in view of our belief that the response to stimulation which occurs when a cell is induced to divide as a result of treatment with various agents is similar to the response to stimulation which occurs generally in various types of irritable protoplasm, we thought that a proper study of the Chaetopterus egg cortex might help us to understand why it is that so many different types of agents can cause an egg cell to undergo division. It is true that in earlier publications an attempt has been made to interpret the initiation of cell division in terms of the colloid chemical theory of stimulation (see Heilbrunn, 1937, 1943, 1952). But when this earlier discussion was written, there was no direct evidence that an egg cell when it was exposed to a stimulating agent behaved like an ameba. The present study is an attempt to study that evidence.

We can say at the outset that our study has been successful from both standpoints. As we shall proceed to show, the cortex of the Chaetopterus egg does behave very much like the cortex of the ameba. And secondly we have found ample support for the view that initiation of cell division can be interpreted in terms of the colloidal theory of stimulation. For the most varied types of stimulating agents do cause both an initiation of division and a liquefaction of the cortex.

As in our earlier work (Heilbrunn, Wilson and Harding, 1951; Wilson, 1951) we estimated the stiffness of the cortex by determining the force necessary to move granules out of it. With the aid of an Emerson hand centrifuge,[2] eggs were exposed to varying centrifugal forces for a minute. Then they were examined under high power of the microscope. Generally speaking, each of us examined ten centrifuged eggs, and from the twenty eggs observed we calculated the percentage of eggs which showed a definite movement of granules from the cortex. We made one series of tests for the eggs in the solutions whose effects we were testing and another series of tests for the control eggs in sea water. The end point was taken as that centrifugal force just sufficient to cause movement of granules from the cortex. In order to get this exact value, we usually resorted to interpolation. For example, if we found that turning the centrifuge 40 times in a minute caused 40% of the eggs to show movement of granules from the cortex, and turning the centrifuge handle 42 times in a minute caused 60% of the eggs to show movement of cortical granules, then the end point was taken as the force developed by turning the centrifuge handle 41 times per minute. No claim is made for the exactness of our values, but in every case repetition of the experiments gave us concordant results. One difficulty in performing the experiments arose from the fact that in the control eggs the rigidity of the cortex kept decreasing during the course of the experiment. In other words, as the eggs aged, the cortex became progressively less rigid. However, this was not a serious difficulty for we made measurements of the control eggs from time to time, so that the value finally recorded was the appropriate value for comparison with that of the experimental eggs.

The results of our studies are shown in Table I. This table does not give the time at which the measurements were made. In almost all cases the effect of the environment on the cortex was rapid, so that probably time did not play much of a role in determining the magnitude or the nature of the effect. However, calcium chloride seems to be rather slow in its action, so that a pronounced effect of the calcium apparently does not occur until about half an hour or longer.

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[2] These hand centrifuges can no longer be purchased from the Emerson Co., but they can be supplied at a reasonable price by Mr. J. A. Appenzeller of the Zoology Department of the University of Pennsylvania.

TABLE I

The effect of various agents on the rigidity of the protoplasmic cortex

Treatment	Centrifugal force in gravities Experimental	Control
Potassium oxalate (70 pts. s.w. + 30 pts. 0.35 *M* K oxalate)	1600	4000
Potassium chloride (0.53 *M*)	1700	4400
Calcium chloride (0.3 *M*)	8400	6400
Sodium hydroxide (pH 9.34)	1200	3200
Ammonium hydroxide (pH 9.65)	600	5100
Hydrochloric acid (pH 3.78)	3900	6100
Ether (2%)	1400	5100
Butyl alcohol (1.5%)	1300	5600
Amyl alcohol (0.5%)	1600	6000
Heat (38° C.)	3200	5400
Cold (0° C.)	3000	4900
Ultraviolet	1100	4000

The table does not give information as to how the eggs were exposed and further detail is necessary. When eggs were placed in solutions containing potassium oxalate, they were washed at least once in the oxalate solution, so as to avoid the presence of too many oxalate crystals. Eggs exposed to potassium chloride solutions were washed several times in the solution; this was to get rid of traces of calcium. In studying the effect of sodium hydroxide, normal NaOH was added to sea water. Solutions of various pH's were tested; pronounced effects were obtained with a solution having a pH of 9.34. Likewise, in studying the effect of ammonium hydroxide, solutions of varying pH were used. We are not prepared to state exactly how much alkalinity is necessary to produce a marked change in the cortex. With ammonium hydroxide, a pH of 8.9 does produce liquefaction, although this is not as pronounced as that which occurs in more alkaline solutions. In experiments with fat solvents we used as high a concentration as we could without destroying the eggs. Thus, for example, in our experiments with butyl alcohol, eggs treated with a 2% solution (by volume) broke up when centrifuged. A 1% solution had little effect on the eggs; a 1.5% solution had an effect without injuring the eggs too greatly. It should be noted that the concentrations of all fat solvents listed in the table are expressed as volume per cent.

In the heat experiments, a temperature of 36° C. had about as much effect as did a temperature of 38° C. In our experiments with cold, we found that moderately cold temperatures were without effect; only when eggs were exposed to temperatures near 0° C. was the cortex markedly affected. In our experiments with ultraviolet, we used a powerful Uviarc lamp. This lamp gives off a whole spectrum of radiations, but we were concerned not so much with determining which wave lengths were effective or how much energy was involved, but rather with understanding what happened to the cortex when the eggs were stimulated by ultraviolet. However, we were careful to exclude the effect of heat. Accordingly, eggs to be irradiated were exposed in Stender dishes immersed in fingerbowls containing cold sea water (at about 10° C.). Under these conditions the sea water in the Stender dishes containing the eggs did not warm up above the room temperature. Eggs were exposed for 4 minutes; the target distance was 9.5 centimeters.

Let us now attempt to answer the first of the two questions we proposed for consideration. Are the properties of the gel in the cortex of the Chaetopterus egg similar to those of the gel in the cortex of the ameba? In ameba, potassium ion markedly decreases the viscosity of the cortical gel and calcium increases it (Heilbrunn and Daugherty, 1932). The gel in the cortex of the ameba tends to be liquefied by oxalate (Heilbrunn and Daugherty, 1933); the indication is that the Chaetopterus cortical gel acts in the same way, although the effect produced by potassium oxalate may have been due to potassium ion. Ether, butyl alcohol and amyl alcohol all tend to cause liquefaction of the Chaetopterus egg, just as they do in ameba (Daugherty, 1937). Cold and heat likewise exert a liquefying effect in both the cortex of Chaetopterus and that of ameba; ultraviolet likewise has a similar effect on both. There are no published data on the effect of alkali and acid on the cortex of ameba, but various workers in the Zoological Laboratory of the University of Pennsylvania have shown in unpublished experiments that alkaline solutions cause a very marked decrease in the viscosity of the ameba cortex just as they cause a drop in the rigidity of the Chaetopterus cortical gel. Indeed, there appears to be only one point of difference between the behavior of the Chaetopterus cortex and the behavior of the cortex in ameba. Acid solutions cause a decrease in the rigidity of the Chaetopterus cortex. On the other hand, unpublished experiments indicate that acids have the opposite sort of an effect on the ameba cortex. On the whole, however, there is a marked similarity in the behavior of the two types of gels. Moreover, Berwick (1951) has shown that a fat solvent like ether causes a release of calcium from muscle brei, and Weimar (unpublished experiments) has found that cold, heat, ultraviolet and potassium all have a similar effect on brei from homogenized frog muscles.

Now as to the second of the questions we proposed. Stimulating agents (heat, cold, electric shocks, ultraviolet radiation) all cause a liquefaction of the cortex of ameba. And as is apparent from Table I they likewise all cause a sharp drop in the rigidity of the cortical gel in the Chaetopterus egg. Moreover, all the agents which stimulate the Chaetopterus egg to divide and begin its development cause a liquefaction of the cortex. Many years ago, Loeb (1901) showed that potassium ions cause an initiation of development in the Chaetopterus egg. We repeated these experiments with isotonic potassium chloride and again found that cleavage was initiated. In one experiment, a 14 minute exposure to a 0.53 molar potassium chloride solution gave 21% cleavage, and a 27 minute exposure gave 34% cleavage. Some of these cleavages look rather normal, but some might not have been true cleavages; it would not have been possible to decide without making cytological preparations.

Alkalies also can cause the initiation of cleavage and development in Chaetopterus. Something of this sort is mentioned by Loeb in his paper, but he is not sure whether the effect is due to alkali or to potassium ions. In one of our experiments in which eggs were kept in a solution of sea water to which sodium hydroxide had been added until the pH was 9.34, 59% cleaved. These eggs had been subjected to the alkali for nearly 5 hours (292 minutes). In another experiment, there was a high percentage of cleavage in eggs left in solutions made alkaline with sodium hydroxide. The pH of these solutions was 8.67 and 8.96. Of all the parthenogenetic agents used by Loeb, the most successful were hypertonic solutions. We therefore were interested in trying to find out whether such solutions caused a liquefaction of the cortex. The evidence that we have indicates that they do. But the tests are complicated by two factors. In the first place, if strong hypertonic solutions are used, the interior protoplasm of the cell becomes so stiff that even vigorous centrifuging does not move the granules in the interior of the cell. Under these conditions the granules of the cortex can not properly be observed, for it is only when there is a hyaline zone free from granules that the cortex stands out as a distinct layer which can readily be studied. In the second place, hypertonic solutions cause a pronounced swelling of the cortical granules.

In our tests when 10 ml. of 2.5 molar sodium chloride was added to 90 ml. of sea water, no great amount of cleavage occurred. However, if 15 ml. of the hypertonic solution was added to 85 ml. sea water or when 20 ml. of the hypertonic solution was added to 80 ml. of sea water, high percentages of parthenogenetic development were obtained. In one experiment eggs were exposed to 15 ml. of 2.5 molar sodium chloride plus 85 ml. sea water for 13, 24, 49, 69 minutes, and then returned to sea water. These exposures gave 34%, 38%, 57%, and 58% cleavage, respectively. In this experiment a centrifuge test was made one minute after the cells were placed in the hypertonic solution. At this time, the cortex of the eggs exposed to the hypertonic solution was decidedly less rigid than the cortex of the control eggs in sea water. However, because of the factors mentioned above, no quantitative measure was possible.

Heat and cold can also cause the egg of the Chaetopterus to cleave. No great attempt was made to discover exactly which exposures gave the best results. In one experiment in which eggs were heated to 36° C. and then allowed to cool slowly, 34% of the eggs cleaved. Exposures to cold did not give such high percentages. In one experiment an exposure to 0° C. for two minutes gave 11% cleavage; in another experiment, eggs exposed to − 0.5° C. for two minutes gave 9% cleavage.

One thing is certain: all the agents which have been shown to cause initiation of cell division and initiation of development in the Chaetopterus egg cause a decrease in the rigidity of the cortex. But not all the agents which cause this change in the cortex are effective in initiating cleavage. This is due to the fact that some of the agents we tried are very toxic, and the eggs are killed by them before there is any chance for development.

The cortex of the Arbacia egg is much more difficult to study than is the cortex of the Chaetopterus egg. The cortical granules in the Arbacia egg are decidedly smaller than the cortical granules in the Chaetopterus egg. This makes them harder to see and it also makes them harder to move by centrifugal force, for according to Stokes' law the force required to move a particle varies as the square of the radius of the particle and thus the force required would be much greater for a small than for a large particle. We have made only a preliminary study of the cortex of the Arbacia egg, but our study has gone far enough to show that it has the same type of structure as the cortex of the Chaetopterus egg. In the Arbacia egg also, the cortex tends to be liquefied by oxalate and by ether. Moreover, just as in the Chaetopterus egg, the Arbacia cortex tends to become less rigid as the egg ages.

Both in the Chaetopterus egg and under some conditions in the Arbacia egg, it is possible to move the cortical granules to one side of the cell. This makes possible a study of the influence these granules may have on the organization of the egg. In earlier centrifuge studies on egg organization it was possible to move only the granules in the egg interior. If, after suitable liquefying treatments, the granules of the cortex are moved also, this may have an important effect on the future development of the egg.

SUMMARY

1. The cortex of the Chaetopterus egg is made more rigid by excess calcium; it tends to lose its rigidity when exposed to oxalate solutions or to potassium ions.

2. The cortex of the Chaetopterus egg is also liquefied by acid, alkali, fat solvents, heat, cold, ultraviolet radiation and hypertonic solutions.

3. In general, the behavior of the Chaetopterus egg cortex is very like that of the cortex of ameba.

4. All agents which have been found to stimulate the Chaetopterus egg to divide cause a liquefaction of the cortex.

5. The results of this investigation lend additional support to the colloid chemical theory of stimulation.

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STUDIES ON BASOPHILIA OF NUCLEIC ACIDS: THE METHYL GREEN STAINABILITY OF NUCLEIC ACIDS[1]

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A number of reports in the recent cytochemical literature have dealt with the use of the basic dye methyl green as a specific and quantitative stain of desoxyribonucleic acid (DNA). Pollister and Leuchtenberger (1949) first demonstrated by photometric methods the high degree of reproducibility of nuclear methyl green staining in mouse liver and discussed the impairment of stainability caused by pretreatment of slides with hot water. Kurnick (1950a) concluded from test tube experiments that methyl green combines specifically with highly polymerized DNA only. He used the stain for quantitative determinations of DNA in fixed histological material (1950b; Kurnick and Herskowitz, 1951) and to measure depolymerase activity of serum (1950c). Vercauteren (1950) and Devreux *et al.* (1951) also tested the dye-binding capacity of DNA solutions under various conditions and concur with Kurnick's opinion. The methyl green stainability of some types of degenerating chromatin was investigated by Leuchtenberger (1950), Klemperer *et al.* (1950) and Korson (1951), and the influence of x-radiation on stainability of nuclei was tested by Moses *et al.* (1951) and Harrington and Koza (1951).

Impairment of methyl green stainability as it was found to occur in some instances, and as it can be induced by treatment of slides with hot water or mild acid hydrolysis, would, according to Kurnick's view, be due to a depolymerization of the DNA. Pollister and Leuchtenberger (1949) have also used the term "depolymerization" to designate an unspecified change in the configuration of the DNA molecule without implying that depolymerization in the chemical sense (breakage of internucleotide linkages) was necessarily involved.

Although methyl green has long been regarded as an exclusive nuclear stain, there are some indications that it does not possess absolute specificity for DNA, since its ability to distinguish DNA from ribonucleic acid (RNA) depends on the type of fixation used: following fixation in acetic alcohol, staining is entirely restricted to nuclei in many types of tissues, while after fixation in formalin, specificity is often lost and cytoplasmic RNA also binds methyl green strongly. Furthermore, in some tissues RNA is methyl green stainable even after acetic alcohol fixation.

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[1] The work reported here started when the author was a Public Health Research Fellow of the National Institutes of Health. At present the author receives support from the University of California Board of Research and from California Cancer Research Funds.

This is notably the case in Nissl substance (as observed by Koenig, and by Swift and Flax, personal communications); we have also found it to occur in the sperm cytoplasm of *Ascaris megalocephala* and in the serous cells of the submaxillary gland of the mouse. In accordance with the concept proposed by Kurnick, it seemed possible that certain types of RNA occur in a highly polymerized state and are therefore able to combine with methyl green. We recorded a hypothesis to that effect in a recent abstract (Alfert, 1951) when we observed that smears of RNA from tobacco mosaic virus, which was known to be highly polymerized, could be stained with methyl green. However, further experiments demonstrated that this hypothesis was inadequate and that other factors, which have not been previously described, strongly influence the methyl green stainability of nucleic acids.

MATERIALS AND METHODS

The staining reaction of nucleic acids in several animal and one plant tissue was investigated. All tissues were fixed in Carnoy's acetic alcohol and paraffin sections of different materials were mounted together to be treated and stained under identical conditions. Several nucleic acid and nucleoprotein model systems [2] were also used; artificial fibers of nucleohistone prepared from calf thymus were fixed, embedded and sectioned like tissues, and drops of solutions were allowed to dry on slides, fixed in acetic alcohol or ethyl alcohol, and stained. The usefulness of the latter staining models is rather limited because variable amounts of the substrate are likely to be lost from the slides during the staining procedure. Only positive results are significant and constitute at best a qualitative test since a reliable comparison of relative staining intensities is not possible.

Methyl green, Natl. Aniline Div., C.I. No. 685, NG26 was purified by chloroform extraction and dissolved in the phenol-glycerin medium used by Pollister and Leuchtenberger (1949) in a concentration of 0.25%. The pH of this solution is 4.5. Materials were stained for 90 minutes at 37° C., rinsed in distilled water, blotted and differentiated for at least 18 hours in tertiary butyl alcohol. This method results in an excellent and, as shown by photometric measurements, very reproducible stain. For histological purposes we found this staining method superior to that proposed by Kurnick (1950b) since staining is more intense and even throughout the section. Both methods are of equal specificity when tested on the same objects.

The methyl green stainability of all substrates was compared to their general basophilia tested with the basic stain azure B. As described by Flax and Himes (1951), this stain permits visualization of DNA and RNA in different colors, blue and purple, respectively. Occasionally other basic stains were used in aqueous and buffered solutions, and the Feulgen reaction was applied (12 minutes hydrolysis in 1 N HCl at 60° C.).

Removability of the stainable substrates by hot trichloroacetic acid and by specific nucleases was used to insure the specificity of the staining reaction for nucleic acids.

A number of treatments which affect basophilia of nucleic acids were applied to fixed materials prior to staining:

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[2] We are greatly indebted to Dr. C. A. Knight as well as to Dr. D. Mazia and members of their laboratories for supplying the nucleic acid and nucleoprotein extracts used in this investigation.

1. Immersion of slides into hot distilled water (90°–95° C.) for 30–60 minutes.

2. Hydrolysis in 1 N HCl at 60° C. for 12 minutes, and immersion in dilute acid (0.01 N HCl) and alkali (0.01 N NaOH) for 24 hours at room temperature.

3. Van Slyke reaction as applied by Ornstein and Flax (unpublished) to remove amino groups of proteins: 10 g. $NaNO_2$ in 45 cc. 50% ethyl alcohol and 15 cc. glacial acetic acid; slides treated for two hours at room temperature.

4. Acetylation of amino groups by acetic anhydride: slides blotted dry out of absolute alcohol and immersed for one hour at room temperature.

A comparison of the relative staining intensity of nuclei was made by means of photometric techniques described elsewhere (Pollister and Ris, 1947; Swift, 1950). The apparatus is similar to that described by Pollister and Moses (1949) and a Beckman B spectrophotometer was used as a light source. The Feulgen reagent was measured at 550 $m\mu$, and methyl green at 630 $m\mu$, slightly off the absorption peak of the dye combined with nucleic acids. Measured values are reported in arbitrary units of dye per nucleus. The tables give mean values and standard errors obtained in several series of measurements.

EXPERIMENTS AND OBSERVATIONS

1. *Cytoplasmic basophilia and staining models*

Plate 1 contains photomicrographs of four different tissues of a mouse, each stained with azure B and methyl green for comparison. The tissues in the first vertical column—liver, pancreas, submaxillary gland and spinal cord—were stained with azure and the presence of high concentrations of RNA can thereby be demonstrated in all of them. The staining picture of the same tissues with methyl green (second column) is quite different, however; in liver and pancreas the methyl green picture is completely equivalent to a Feulgen preparation of the same material, only nuclei stain and the cytoplasm remains unstained. In the submaxillary gland, however, the cytoplasmic RNA of serous cells combines with methyl green and the same is true for Nissl substance in the motor neurons of the spinal cord. Comparing the two photographs of motor neurons, it is evident that the strongly azurophilic nucleolus does not stain with methyl green. Figure 9 on Plate II is a methyl green preparation of a fertilized egg of Ascaris. In addition to the egg chromosomes and the small sperm nucleus, the whole body of the amoeboid sperm stains strongly with methyl green.

Thus there exist clear-cut differences among ribonucleic acids in different tissues with respect to their ability to bind methyl green. Using model systems of different nucleic acid and nucleoprotein solutions, we were unable to find qualitative differences in stainability. The following systems were investigated:

1. Calf thymus nucleohistone.

2. Calf thymus nucleohistone after irradiation with a dose of 100,000 r (Himes and Alfert, unpublished).

3. Nucleoprotein from sea urchin sperm.

4. 0.1% solution of DNA from chicken erythrocytes.

5. 0.1% solution of yeast RNA (Schwarz).

6. 0.1% solution of tobacco mosaic virus RNA.

7–10. Solutions 3–6 after heating for one hour in a bath of boiling water.

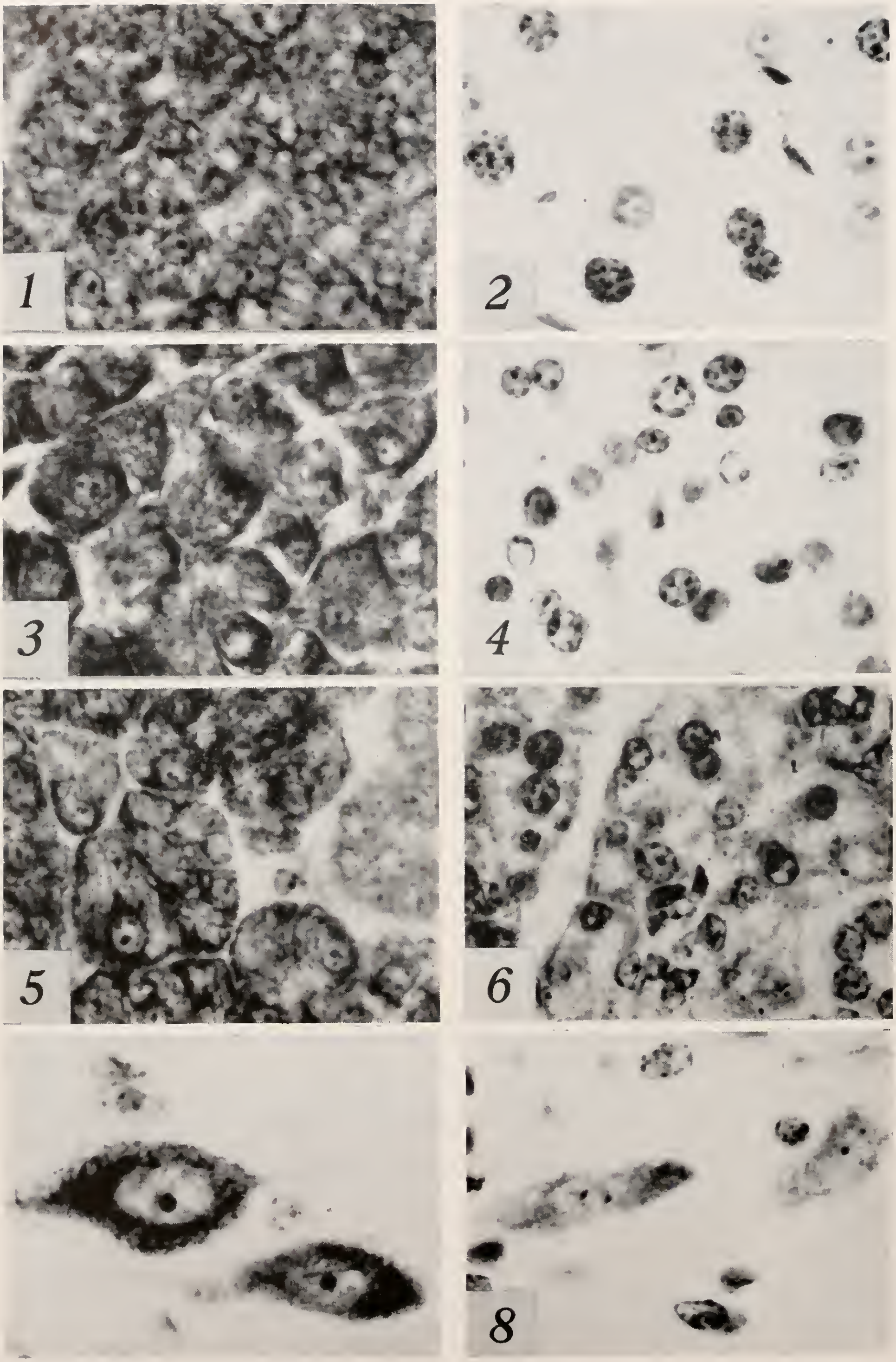

Plate I. Mouse tissues fixed in Carnoy's acetic-alcohol; 10 μ sections, × 750. (Photomicrography by Mr. Victor Duran.)

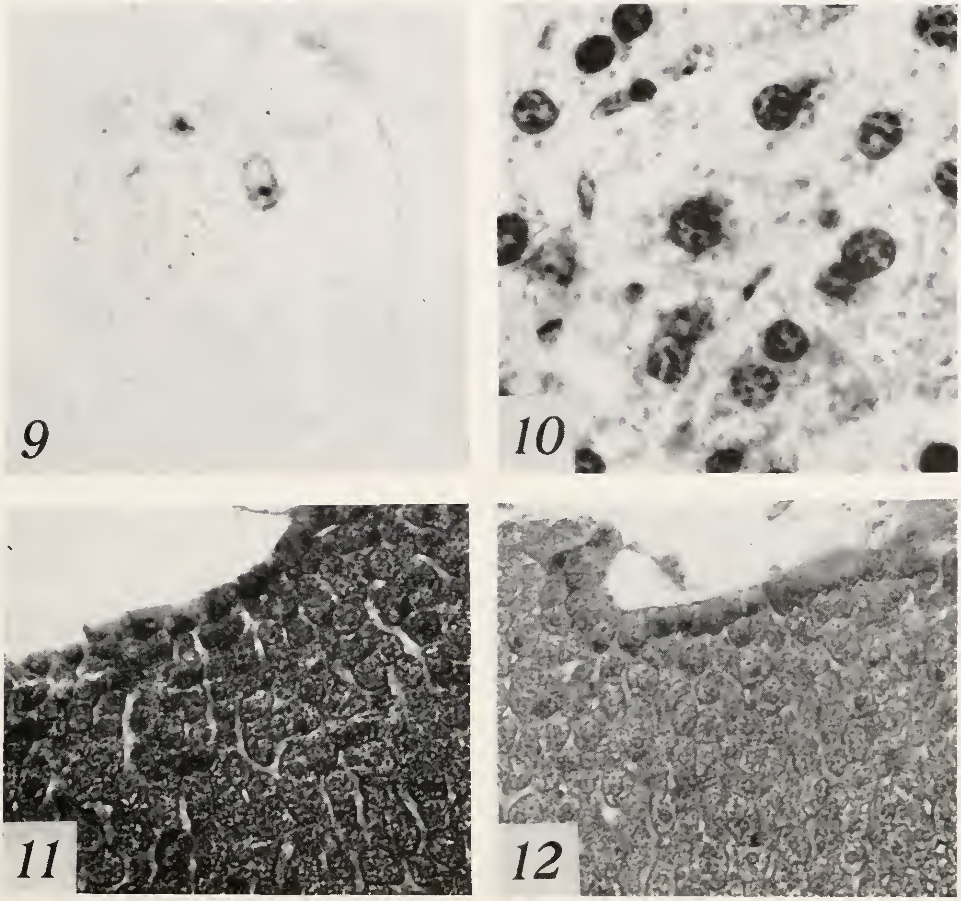

Plate II. (Photomicrography by Mr. Victor Duran.)

Figure 9. Fertilized egg of *Ascaris megalocephala*, Carnoy's, 15 μ section, × 750; stained with methyl green; Wratten F filter.

Figure 10. Mouse liver, as in Figure 2, but first acetylated and then stained with methyl green.

Figures 11 and 12. Mouse liver, × 220; stained with fast green at pH 2; Control (Fig. 11), and after acetylation (Fig. 12).

All these materials stained with methyl green. Fixed smears of isolated cell particulates exhibit staining differences which correspond to those found in histological sections: liver nuclei stain with methyl green and azure, while mitochondria and microsomes stain only with azure but not with methyl green.

If lack or presence of methyl green stainability is not a property of the nucleic acid itself, as these model experiments seem to suggest, a possible clue for the

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Figures 1 and 2: liver; Figures 3 and 4: pancreas; Figures 5 and 6: submaxillary gland; Figures 7 and 8: motor neurons. Odd numbered figures: stained with azure B; Wratten G filter. Even numbered figures: stained with methyl green; Wratten F filter.

explanation of the observed staining differences in tissues is perhaps provided by the effect of formalin. As mentioned above, fixation in strong formalin (or treatment of Carnoy-fixed material with 50% formalin for three hours) causes loss of methyl green specificity for nuclei. Formaldehyde is known to combine with amino groups of proteins. This reaction could result in making available, for combination with the dye, nucleic acid phosphate groups previously masked by the protein. This hypothesis was tested by subjecting sections to two chemical procedures known to affect amino groups of proteins, the Van Slyke reaction and acetylation by acetic anhydride (see Olcott and Fraenkel-Conrat, 1947). Under the conditions used, these two treatments produce similar results. Their immediate effect consists in a decrease of acidophilia which can be demonstrated by comparing sections stained with fast green at pH 2 before and after treatment (Figs. 11 and 12). The same effect was described by Monné and Slautterback (1951) who used similar procedures. While these reactions result in a loss of some basic groups available for combination with acid dyes, they may at the same time affect the methyl green stainability of nucleic acids; Figure 10 demonstrates that RNA in liver cytoplasm becomes methyl green stainable and that nuclei stain more intensely after acetylation. The treatments also cause the nucleolus in neurons to become methyl green stainable and enhance the stainability of Nissl substance and submaxillary gland cytoplasm and nuclei. Smears of isolated liver mitochondria and microsomes also become methyl green stainable after either treatment. The stainability of ribonucleic acid with azure in any of the mentioned materials is not increased further by these treatments and they do not affect the Feulgen stainability of nuclei.

For unknown reasons the strongly basophilic cytoplasm of the exocrine cells in pancreas does not become methyl green stainable after acetylation or Van Slyke reaction. This probably reflects a fundamental difference in the structure of the nucleoproteins as compared to those of liver cells. Differences in the nucleoproteins of various tissues with respect to their solubility have been described by Koenig and Stahlecker (1952).

Digestion of sections with trypsin in aqueous solution as described by Kaufmann *et al.* (1950) does not produce cytoplasmic methyl green stainability in liver and pancreas. Since the azure stainability was decreased after trypsin digestion, it appears that the enzyme removed the whole nucleoprotein complex in these materials.

The qualitative observations on cytoplasmic staining reported so far indicate that RNA in different tissues may be methyl green stainable or not, and suggest that this is a matter of absence or presence of protein interference. The chemical treatments used bring out further differences in that they may cause an unmasking of stainable groups in some materials but not in others.

2. *Nuclear basophilia*

As far as nuclear staining is concerned, our own data confirm those of Pollister and Leuchtenberger (1949) relating to the reproducibility of the methyl green procedure. Samples of 20 photometric measurements of the amounts of methyl green bound by diploid nuclei of the mouse pancreas were measured on different slides, prepared and stained at different times. The mean values of methyl green content per nucleus thus obtained were quite similar: 100 ± 4, 104 ± 4, and 111 ± 4 (see Table III).

A comparison of the methyl green and Feulgen stainability of tetraploid and diploid nuclei in the liver and pancreas of the mouse was also made and the data are presented in Table I.

Recent work by Ris and Mirsky (1949), Swift (1950) and Leuchtenberger, Vendrely and Vendrely (1951) has demonstrated that photometric Feulgen dye determinations, if done correctly, provide a reliable measure of the DNA content and degree of ploidy of a nucleus. The present data indicate that this is also true for methyl green staining in these two tissues of the same animal, since very similar Feulgen/methyl green ratios are obtained. The average Feulgen/methyl green ratio of these four types of nuclei is therefore a characteristic constant of 2.56 ± 0.07 for these tissues. Measurements conducted under the same conditions on 25 onion root tip nuclei produced a very different Feulgen/methyl green ratio of 1.45 ± 0.03. This indicates that the dye-binding characteristics of this material are not the same as in the previous case.

TABLE I

Amounts of DNA in arbitrary units of Feulgen dye and methyl green in different nuclei*

	Type of nucleus	Number measured	Average amount Feulgen	Average amount methyl green	Ratio Feulgen/methyl green
Mouse pancreas	2n	25	277± 6	100±4	2.77
	4n	15	533±16	212±8	2.52
Mouse liver	2n	15	275± 6	112±4	2.46
	4n	25	597±11	239±6	2.50
					average 2.56±0.07
Onion root tip		25	—	—	average 1.45±0.03**

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* To facilitate comparison, the methyl green value for diploid pancreas nuclei obtained in one experiment is set equal to 100; all other values are proportional to that.

** Calculated on basis of optical densities measured consecutively on the same nuclei stained first with methyl green and then by the Feulgen reaction. (The Feulgen/methyl green ratio of mouse tissue nuclei calculated on basis of average optical densities is 2.47±0.05, very similar to that given above which is calculated from average amounts of dye per nucleus.)

It remains to be determined whether a change in the Feulgen/methyl green ratio of a nucleus can be correlated with any particular process—such as depolymerization of the nucleic acid.

a) Treatment with acid and alkali

Taft (1951) criticized the depolymerization concept on basis of the following experiment: soaking of sections in dilute acid and alkali (which he assumed would cause depolymerization of the DNA) resulted in a decreased Feulgen stainability without affecting methyl green stainability of nuclei. The only conclusion that can be drawn from such an observation is that methyl green, as it was used in this case, did not stain DNA specifically at all. We have repeated this experiment and obtained different results: treatment with dilute HCl (pH 2.1) for 24 hours at room temperature did not affect the Feulgen reaction but increased methyl green stainability noticeably. The latter may be due to extraction of histone by HCl (Kurnick, 1950b). Treatment with dilute NaOH (pH 8.1, 24 hours, room temperature) had a profound effect: nuclei were swollen, vacuolated and in various stages of disintegration. Strands of Feulgen positive and methyl green stainable chromatin were scattered throughout the cytoplasm. Although chromatin had been partially dissolved and still retained methyl green stainability, this observation does not constitute a test of the depolymerization hypothesis, since alkali at low temperature can decrease viscosity without depolymerizing DNA (see discussion by Davidson, 1950).

b) Enzymatic digestion

The action of desoxyribonuclease results in depolymerization and loss of DNA from sections. The effectiveness of enzymatic digestion depends on the type of fixation and also differs in various materials, depending probably greatly on the compactness of the nuclei. Under the conditions which we used, interphase nuclei in many mouse and Ambystoma tissues were Feulgen and methyl green negative after digestion for 40 minutes at room temperature. Some nuclei in sections of Ambystoma spleen, however, retained Feulgen and methyl green stainability after being exposed to enzyme for as long as 90 minutes at room temperature. Used at low temperature and for a shorter time, the enzyme decreased methyl green stainability more rapidly than Feulgen stainability in mouse liver nuclei (Table II). This effect was also noted by Leuchtenberger, Himes and Pollister (1949).

TABLE II

Effect of desoxyribonuclease (0.15 mg./cc. at pH 7 for 30 minutes at 4° C.) on stainability of tetraploid mouse liver nuclei

	Number measured	Average amount Feulgen	Average amount methyl green	Ratio Feulgen/methyl green
Control	25	597±11	239±6	2.50
After DNA-ase	15	457±15	142±11	3.72
Reduction by enzyme		23%	41%	

c) Effect of hot water and hot acid hydrolysis

Treatment of sections with hot water and Feulgen hydrolysis (1 N HCl at 60° C. for 12 minutes) removes methyl green stainability of DNA. However these treatments also affect the azure stainability of DNA in a similar way; the effect produced is therefore likely to have a more general basis than the peculiar specificity of methyl green. We observed that basophilia of nucleic acid can be partially restored in these cases by subjecting sections to acetylation, the Van Slyke reaction or trypsin digestion. Photometric measurements on several experimental series are presented in Table III.

All determinations were made on diploid pancreas cells in which the treatments used do not produce cytoplasmic staining which would interfere with nuclear measurements. Acetylation or Van Slyke reaction initially increase nuclear stainability approximately 50%. Exposure to hot water produces a small but significant drop of 15% of the Feulgen values while methyl green stainability is reduced to the level of the surrounding cytoplasm (about 95% transmission of the incident light). Following hot water treatment either of the protein reactions will restore stainability to an extent at least as great or greater than the initial increase it produces in unheated slides; and if the protein reactions precede hot water treatment they protect from loss of stainability to the same extent. At any stage of these treatments nuclear methyl green stainability is removable by desoxyribonuclease but resistant to ribonuclease. The azure stainability of nuclei appears to follow the same pattern in the course of these treatments but loss of stainability is never complete. Photometric measurements were not made. Sections of nucleohistone fibers also show similar effects but are more resistant to loss of basophilia than interphase nuclei; they show a less pronounced decrease after hot water treatment and remain methyl green stainable even after Feulgen hydrolysis.

TABLE III

Effects of various treatments on methyl green and Feulgen stainability of 2n mouse pancreas nuclei; each value is a mean of 20 measurements

	Methyl green			Feulgen
	Exp. No. 1	Exp. No. 2	Exp. No. 3	Exp. No. 1
Control	100±4	104±4	111±4	277±6
After acetylation	156±8	—	161±3	267±6
After Van Slyke	—	142±4	140±4	—
After hot water	0	0	0	234±4
After hot water and acetylation	86±4	—	68±4	—
After hot water and Van Slyke	—	85±4	71±3	—
After acetylation and hot water	—	—	69±3	—

DISCUSSION AND CONCLUSIONS

It is necessary to consider the validity of various model systems and test tube experiments which have been used to investigate the mechanism of methyl green staining. Obviously any model system represents a simplification of the very complex conditions that obtain in tissues and the observations made on such models must be interpreted with great caution. The conditions under which the model system is tested will also influence the results. This is demonstrated by the data reported by Kurnick (1950a); in one experiment the dye-nucleic acid complex was precipitated with alcohol and ribonucleic acid was found to combine with an appreciable amount of methyl green. When lanthanum was used to precipitate the dye-NA complex, only highly polymerized DNA retained methyl green. Since staining of tissues is not ordinarily performed in presence of lanthanum, the significance of this observation with respect to the histological use of the stain cannot be evaluated.

In our own experiments the various model systems were stained in the same way as sections of fixed tissues. Under these conditions the dye was found to be unable to distinguish RNA from DNA. Although this constitutes no definite proof, it suggests that the specificity of methyl green for DNA which undoubtedly exists in many tissues is not primarily due to an innate inability of the dye to combine with RNA. Staining experiments on tissues furthermore demonstrate that stainable groups in nucleic acids are made available for dye combination by treatments which are known to affect proteins in definite ways. At this point it is not yet possible to suggest a definite mechanism for the protein interference, since removal of amino groups could affect the nucleoprotein in a number of ways. We know from the work of Kaufmann *et al.* (1951), Mirsky and Ris (1951) and Pollister *et al.* (1951) that masking of stainable groups is a general phenomenon which may affect the staining picture obtained with various dyes. Methyl green seems to differ from other basic dyes mainly in being more sensitive to protein interference.

To account for the behavior of methyl green, Kurnick (1950a) assumed the operation of stereochemical factors. On the basis of *in vitro* experiments he claims that all triphenylmethane dyes with two methyl amino groups, such as methyl green, ethyl green and malachite green, exhibit the same type of selectivity. However one sample of Grübler's malachite green which we tested as a tissue stain did not behave like methyl green : it stained liver cytoplasm as well as nuclei and produced a staining picture similar to the nonspecific methyl violet. Pollister and Leuchtenberger (1949), on the other hand, have pointed out that methyl green carries two positive charges and may therefore require phosphate groups at definite spacings for stable combination. Adopting this view for a possible explanation of methyl green selectivity, we may assume that ribonucleoprotein complexes in many tissues are of a type in which the necessary phosphate groups are masked by protein; but in other cases, such as Nissl substance, methyl green is able to combine either because of the different nature of the complex or because of the presence of some relatively "free" RNA.

DNA in nuclei normally combines to a definite extent with methyl green, resulting in a characteristic Feulgen/methyl green ratio. Changes in this ratio can be brought about in a number of ways and impairment of methyl green stainability is not necessarily related to depolymerization. Even the effect of desoxyribonuclease cannot be interpreted in a clear-cut way. The enzyme may simply remove certain exposed groups necessary for methyl green combination at a faster rate than the many more numerous nucleotides which contribute to the Feulgen reaction. Treatment with hot water and Feulgen hydrolysis affect basophilia of DNA in general and the observations suggest that masking of stainable groups is at least partially responsible. A similar conclusion was reached by Herrmann *et al.* (1950) who found that the toluidine blue binding capacity of RNA is impaired when the nucleic acid is heated in the presence of protein. They attribute this effect, which was observed *in vitro,* to the masking of staining sites by basic groups of protein which had become exposed in the course of heating. In our experiments we have never succeeded, however, in restoring maximum stainability in heat-treated sections. This may be due to a low efficiency of the protein reactions under the conditions used; since there is some loss of Feulgen stainability in the course of heating it is also possible that a small loss of the Feulgen reactive material is responsible for a much greater loss of methyl green stainability. The mammalian nuclei which we used have a notoriously low RNA content and staining of nuclear RNA can hardly play an important role in this case. This is borne out by our observations on the effect of digestion by nucleases. However in other types of material staining of nuclear RNA may be an important variable.

For histological purposes methyl green is often used in combination with pyronin, the well known Unna-Pappenheim mixture. This can be used as a satisfactory qualitative method to distinguish RNA and DNA in many tissues. As a precise and quantitative histochemical technique for DNA determinations, methyl green is much less satisfactory than the Feulgen reaction which appears to be less subject to modifying influences. In combination with the Feulgen reaction, methyl green is a useful tool which serves to characterize a particular nucleoprotein complex in terms of a Feulgen/methyl green ratio. Changes of this ratio are of interest but they cannot be interpreted in a precise way.

SUMMARY

1. The capacity of nucleic acids to combine with basic dyes under various conditions was investigated by means of fixed tissues and model systems. Observations and experiments suggest that the specificity for nuclear DNA which methyl green exhibits in many, but not all, tissues is caused by blockage of stainable groups of ribonucleic acid by protein.

2. Nuclear staining with methyl green is also subject to modification by changes in the degree of protein interference, and impairment of stainability cannot be correlated unequivocally with depolymerization of DNA.

3. Since variable degrees of RNA staining, differences in the extent to which DNA staining is affected by protein interference, and possibly changes in the degree of polymerization of the DNA may all influence the staining intensity of nuclei, methyl green staining is not a very suitable method for quantitative determination of DNA in cell nuclei.

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THE NEUROSECRETORY SYSTEM OF BRACHYURAN CRUSTACEA [1, 2]

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Physiological evidence has accumulated which no longer favors the sinus gland as the site of formation of the molt-inhibiting hormone of Crustacea (Bliss, 1951, 1952; Frost, Saloum and Kleinholz, 1951; Havel and Kleinholz, 1951; Passano, 1951a, 1951b, 1952a, 1952b; Travis, 1951a, 1951b; Welsh, 1951). The present studies, designed originally to correlate anatomical relationships of the neurosecretory organs in the brachyuran, *Gecarcinus lateralis* (Fréminville), with the molt and respiratory data recorded on this animal (Bliss, 1951, 1952), include comparative observations on a variety of crabs. It has become clear that assignment of crustacean endocrine functions primarily to the so-called sinus glands has been an over-simplification of a very complex situation.

MATERIALS AND TECHNIQUES

Specimens of *Gecarcinus lateralis* were shipped periodically to Cambridge from Bermuda. Most other crabs included in this paper were studied in Bimini, B. W. I., while the first author was a Guest Investigator at the Lerner Marine Laboratory of the American Museum of Natural History.

The characteristic bluish-white hue of the living sinus gland was first noted by Brown (1940), his diagrams of several decapod eyestalks depicting not only the sinus gland but its large bluish-white nerve as well. Both sinus gland and nerve are visible when eyestalks of *Gecarcinus* are dissected either in sea water or in a suitable perfusion fluid. It is possible, after removal of the connective tissue sheath surrounding the ganglia, to trace numerous bluish-white nerve tracts that converge and enter the sinus gland. An intense spot of light reveals the deeper of these tracts.

Accentuation of nerve tracts and bluish-white globules, which have proved to be groups of neurosecretory cells, occurs in eyestalks and brain when a preparation is permitted to stand for some time in the beam of a microscope lamp. Increase in salt concentration due to evaporation of perfusion medium causes tracts and globules already visible to become whiter and others hitherto unseen to be apparent. This effect is accomplished more rapidly by use of concentrated sea water or hypertonic perfusion fluid. Addition of a drop or two of glycerol, which clears the tissue, further intensifies nerve tracts and globules. Careful dissection exposes these tracts as groups of brilliant white fibers.

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[2] A portion of this work was done at the Lerner Marine Laboratory of the American Museum of Natural History, Bimini, B. W. I.

A modification of the conventional methylene blue technique for nerve differentiation has proved useful. The eyestalk ganglia and brain, stripped of their connective tissue sheath, are placed in the well of a depression slide, containing about 20 drops of perfusion medium and two drops of 0.2% methylene blue. After several minutes the preparation is examined against a black background in the same amount of perfusion medium and two drops of glycerol. Alternate staining and clearing yields a preparation in which nerve cells appear deep blue and nerve tracts white with blue edges. Since it is known that both neurosecretory and other

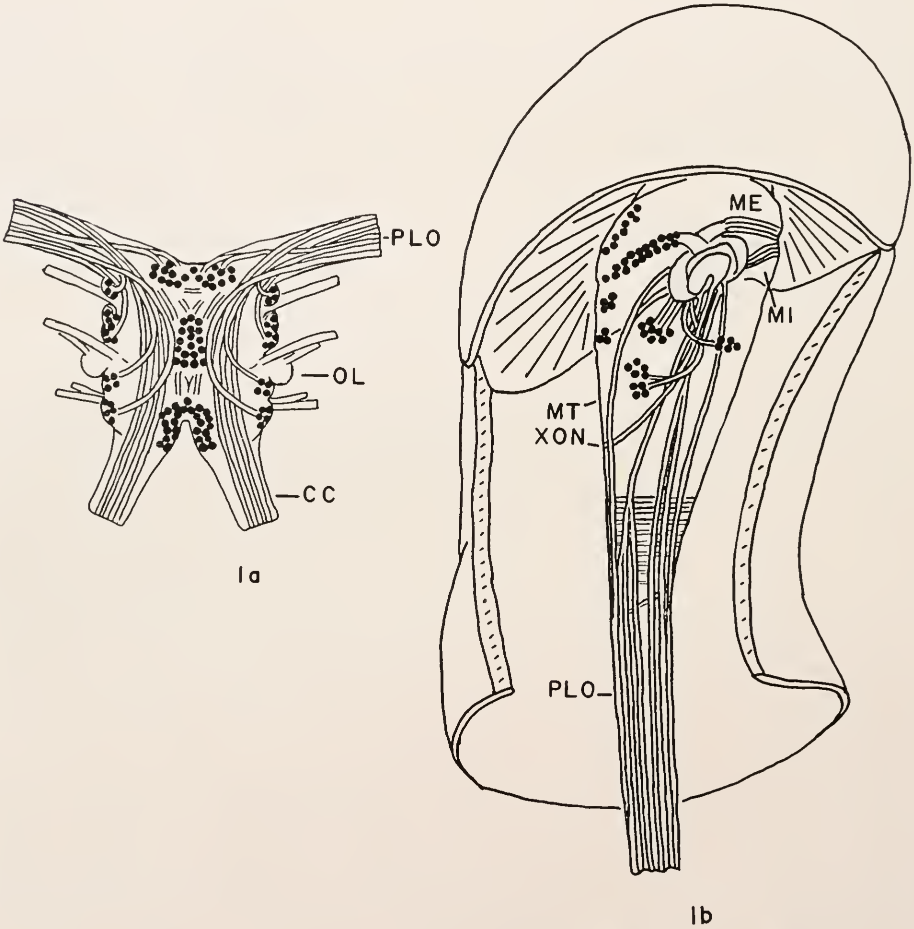


FIGURE 1. Brain (a) and right eyestalk (b) of *Gecarcinus lateralis*, shown in dorsal view when eyestalk is resting in orbit. Circles give location of neurosecretory cell groups. One medial and two lateral groups on the ventral (anterior) face of the brain are not shown. Double lines indicate some of the nerve fiber tracts. Stippled area represents sinus gland. Brain and eyestalk are drawn to the same scale. A considerable portion of the connecting peduncle (PLO) has been omitted. Actual length of adult *Gecarcinus* eyestalk: 0.6–0.7 cm. PLO, peduncle of the optic lobe; OL, olfactory lobe; CC, circumesophageal connective; MT, medulla terminalis; MI, medulla interna; ME, medulla externa; XON, nerve from x-organ.

ganglionic cells accept the stain, these two cell types are then spatially separated by determining in serial sections their relative locations.

Often unstained preparations are best observed in a depression slide against a background of black, white or yellow.

When serial sections are made, tissues are fixed 8 to 12 hours in Helly's, cleared in cedar oil, cut at 6 micra, and stained in chrome-hematoxylin and phloxin, according to Gomori (1941). It has been found that direct immersion of the sections in chrome-hematoxylin without preliminary mordanting in Bouin's results in extraordinarily distinct nerve fibers, whereas after pre-treatment with Bouin's, nerve fibers are relatively indistinct but secretory granules are clearly differentiated.

Observations

Figure 1b shows the right eyestalk of *Gecarcinus lateralis*, as seen in dorsal view with eyestalk in resting position. On casual examination the sinus gland of a freshly-made preparation seems to be innervated by one thick, bluish-white nerve, which is formed at a point proximal to the gland by a branch leading from the ventral portion of the medulla terminalis and one coming from the peduncle of the optic lobe. With application of the several techniques described in the previous section, this nerve is resolved into a number of separate fiber tracts, which intertwine in tortuous fashion as they approach their terminus. Apparent now are many other bluish-white tracts, leading from globules arranged in groups over the surface of the three inner optic ganglia.

The large branch approaching the sinus gland from the proximal ventral portion of the medulla terminalis is the nerve which has been found in crabs by Passano (1951a, 1952a, 1952b) and Bliss (1951, 1952) and in crayfish by Durand (personal communication) to connect the x-organ with the sinus gland. X-organ cells and the nerve which they form are probably identical with the beta cells and the sinus gland nerve of Enami (1951). Bliss and Passano have independently concluded that sinus gland hormone is produced in the x-organ and transported by way of this nerve to the sinus gland. Such a concept is in harmony with that developed for the hypothalamo-hypophyseal system in vertebrates (Scharrer and Scharrer, 1944; Bargmann and Scharrer, 1951; Scharrer, 1952; Palay, 1943, 1945; Smith, 1951), and for the intercerebralis-cardiacum-allatum system in insects (Scharrer and Scharrer, 1944; Scharrer, 1953; Thomsen, 1952).

In Figures 2 and 3 it is shown that, although axons of x-organ cells do form eventually a nerve leading to the sinus gland, the manner in which this is accomplished is quite indirect. The x-organ is composed of two groups of cells, axons of one group forming a tract which descends at once towards the optic lobe peduncle, those of the other group partially circling the medulla terminalis before they head toward the peduncle. Fibers from both tracts now spiral within the peduncle, re-gather in two groups, ascend again to the peduncle, and proceed towards the sinus gland as one large nerve. Evidence for this indirect route has been obtained from glycerine-cleared preparations, both unstained and stained with methylene blue, and from serial sections.

This devious pathway may cause wonder unless it is remembered that the third and fourth optic ganglia are parts of the brain which have, in the course of evolution, been drawn out into the eyestalk. Still pointing towards the center of the brain, as do axons of many neurosecretory cells within the brain, developing x-organ fibers must turn sharply to reach the sinus gland. Re-orientation may be facilitated by a circling of the optic lobe peduncle. Incidentally there results an increase in the space available for storage of secretory material. Where nerve fibers circle and turn distally towards the sinus gland, secretory material accumulates in such amounts that the axons are almost filled with basophilic granules. This may result from reduced rate of movement in these regions. According to E. Scharrer (personal communication), in vertebrates, likewise, secretory material accumulates at axonal bends and turns.

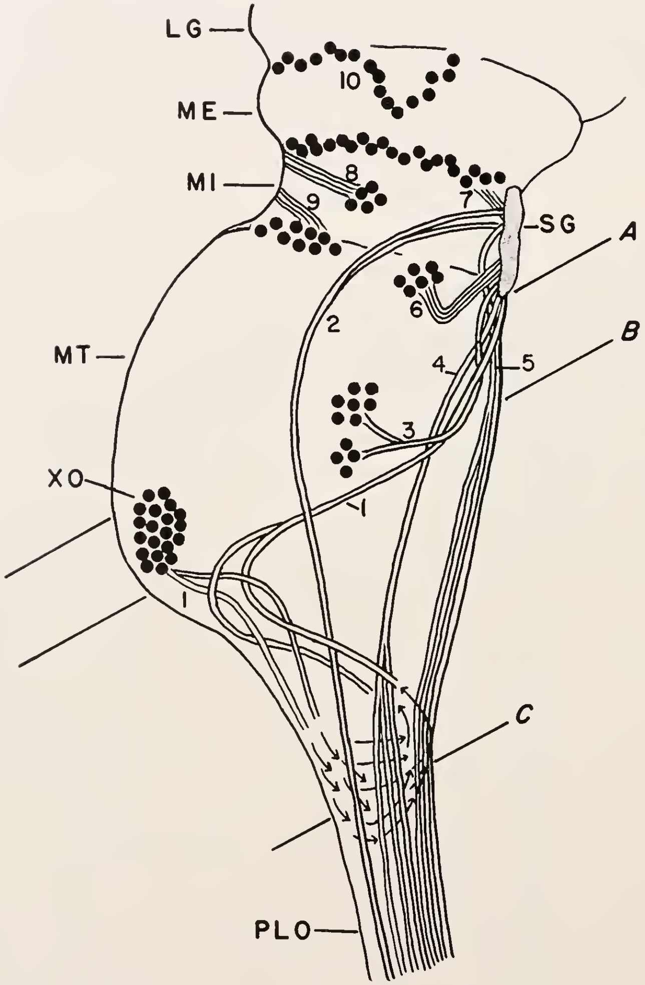


FIGURE 2. The excised right eyestalk ganglia of *Gecarcinus lateralis*, anterior aspect. Circles give location of neurosecretory cell groups. Double lines indicate nerve fiber tracts, which are numbered as in Figure 3. A, B, C designate the three planes of section shown in Figure 3. XO, x-organ; SG, sinus gland; LG, lamina ganglionaris; other abbreviations as in Figure 1.

The original observations that a nerve connects x-organ and sinus gland remain uncontested. The riddle of where the sinus gland nerve runs when, as has been reported often in the literature, it is lost in the medulla terminalis, is solved. This nerve has no identity as such before the point where the peduncle-circling fibers converge.

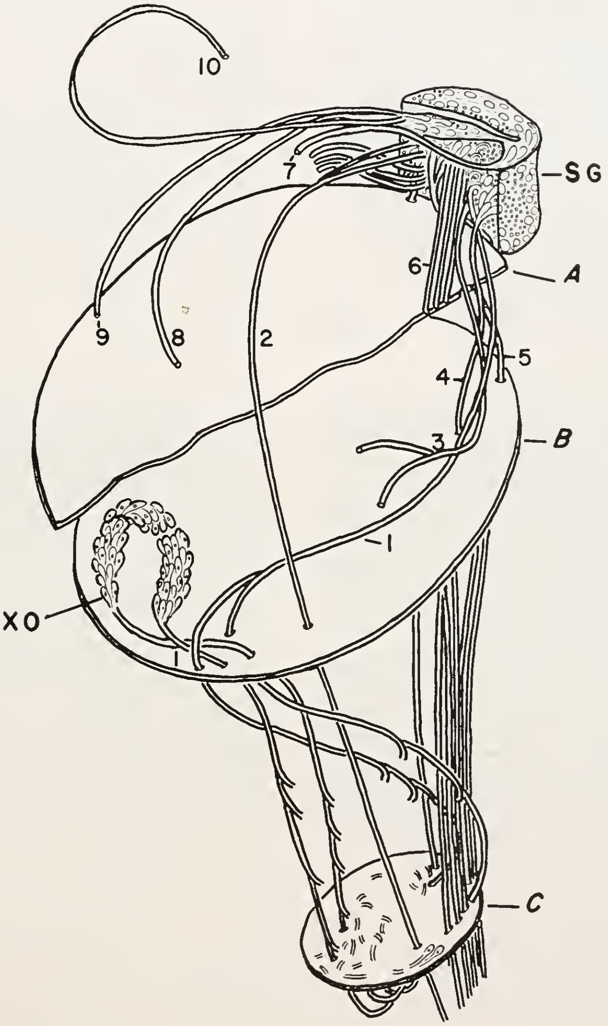
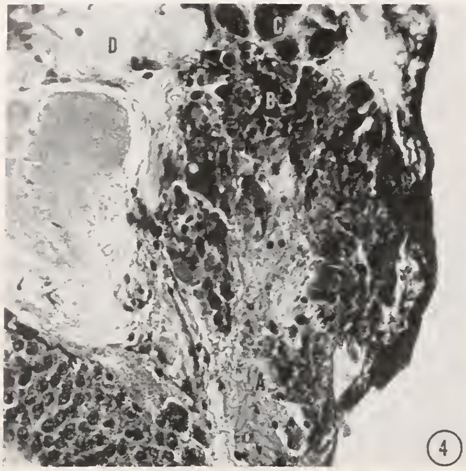
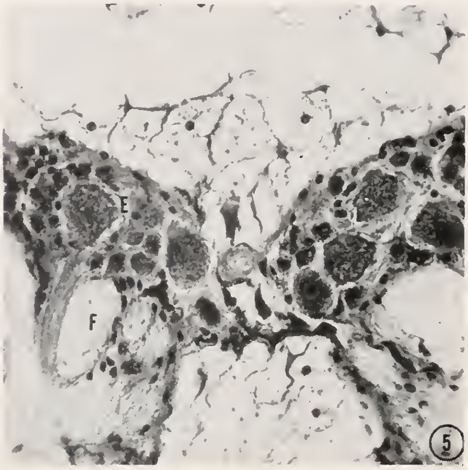
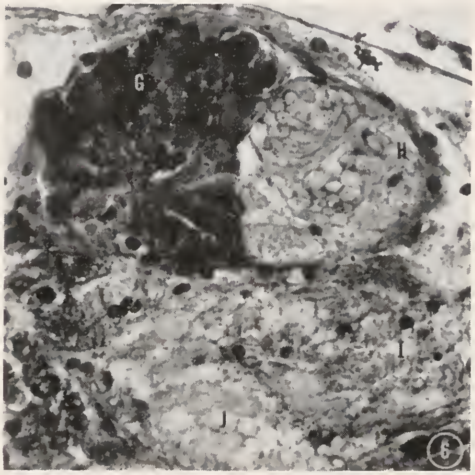


FIGURE 3. Schematic diagram of the x-organ, sinus gland, and fiber tracts of the right eyestalk of *Gecarcinus lateralis*. Fiber tracts and planes of section are designated as in Figure 2. XO, x-organ; SG, sinus gland; 1 (= XON in Figure 1), nerve from x-organ.

Several groups of bluish-white globules observed proximal to the sinus gland in the living preparation and other groups lying distally (Figs. 1b and 2) are recognizable in chrome-hematoxylin-phloxin stained serial sections as masses of neuro-







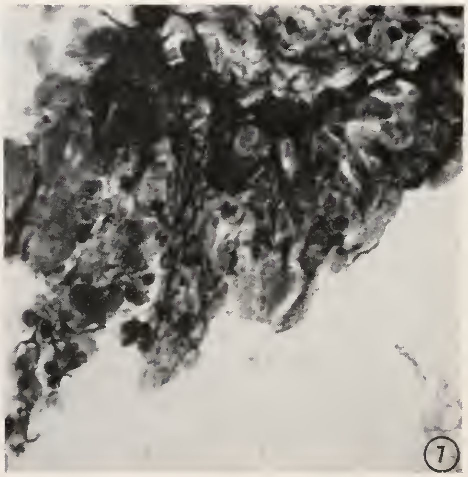


FIGURE 4. Longitudinal section through the sinus gland (right eyestalk of *Gecarcinus lateralis*) in the region where fibers of the x-organ nerve (A) fan out distally to form a sinus gland lobe which has the appearance of an inflorescence. The swollen endings (B) of x-organ axons contain darkly staining secretory material. Distal to the sinus gland are neurosecretory cells (C) and coarse nerve fibers (D) from neurosecretory cells of the medulla interna and medulla externa. Magnification: 200 ×.

FIGURE 5. Two medial frontal groups of neurosecretory cells on the dorsal (posterior) face of the brain of *Gecarcinus lateralis*. In one cell (E) secretory granules fill the axon as well as the cell body. Next to this cell is a large coarse-fibered nerve tract (F) which can be traced in subsequent sections into the optic lobe peduncle. Magnification: 200 ×.

FIGURE 6. Cross section, left eyestalk of *Gecarcinus lateralis*. Included are a small portion of the sinus gland (G), the x-organ nerve fiber tract (H), and fiber tracts (I, J) formed from axons of neurosecretory cells of the brain and medulla terminalis. In x-organ fibers in particular, secretory granules can be seen just inside the fiber membrane. Many granules are visible within the sinus gland. Magnification: 400 ×.

FIGURE 7. A regenerated sinus gland from the right eyestalk of a specimen of *Gecarcinus lateralis* seven and one-half months after both sinus glands had been removed. The regenerated

secretory cells. Coarse lilac-colored axons link cell bodies with the sinus gland. Figure 4 is a longitudinal section showing these coarse axons, the sinus gland, and distal to it large neurosecretory cells. These cells contain concentrically arranged fine granules and irregular inclusions, similar to those described by Hanström (1947) for the cells of the x-organ in Natantia.

Although granules can be seen along an axon for some distance from the secretory cell body, they soon become less numerous or disappear entirely, to appear again in great numbers just before the fiber reaches the sinus gland. The intermediate parts of the fiber contain homogeneous material which stains lilac with chrome-hematoxylin-phloxin. A somewhat analogous situation has been observed in vertebrate hypothalamic-posterior lobe preparations (E. Scharrer, personal communication).

Lying on the surface of the brain (Fig. 1a) are many more neurosecretory cell groups, appearing as collections of bluish globules in the living specimen and as grape-like clusters in serial sections. Figure 5 is a photomicrograph of two medial frontal groups lying on the posterior face of the brain. These neurosecretory cells have the same general characteristics as cells of the insect pars intercerebralis (Scharrer and Scharrer, 1944) and of the vertebrate hypothalamus (Bargmann and Scharrer, 1951; Smith, 1951). They are indistinguishable from neurosecretory cells of the eyestalk of *Gecarcinus* and, like the latter, contain concentrically arranged granular inclusions which stain blue-black with chrome-hematoxylin.

Bluish-white tracts, which appear in serial sections as groups of coarse lilac nerve fibers, run in the living preparation from the globular clusters of the brain into the optic lobe peduncle. Other whitish tracts and, in serial sections, other coarse lilac fibers enter the brain from the circumesophageal connectives and continue out the optic lobe peduncle. Since the thoracic ganglionic mass of *Gecarcinus,* like that of *Sesarma* (Enami, 1951), contains many giant neurosecretory cells, the sinus gland in the eyestalk of a crab appears to be the terminus for axons originating as far away as the thorax. Clearly necessary is a reconsideration of the structure and function of the sinus gland.

Figure 6 shows in cross section the sinus gland and the nerve from the x-organ. Lying just within the membrane of each nerve fiber are granules and granular aggregates, strikingly similar to those found within the cell bodies of these axons and within the sinus gland. In Figure 4 this nerve is seen to be not merely entering but actually forming a portion of the sinus gland. Its fibers, gradually expanding as they terminate to produce the effect of an inflorescence, contain within their bulbous endings acidophilic secretory material and basophilic granules. To the upper left of the sinus gland, axons from neurosecretory cells of the medulla externa approach laterally, to form another section of the sinus gland. Numerous microscopic sections indicate that the sinus gland of *Gecarcinus* is a mass of swollen nerve fiber endings, grouped into lobes according to the fiber tracts from which they arise. This has been represented schematically in Figure 3.

Support for this concept of an organ which previously had been considered glandular in its own right came from regenerated sinus glands. Five weeks after

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structure is characterized by small size and abnormal position but has essentially normal staining properties. Magnification: 400 ×. All photomicrographs made from tissues fixed in Helly's, cut at 6 micra, and stained with chrome-hematoxylin-phloxin.

bilateral sinus gland removal the eyestalks of three crabs contained no sign of sinus glands in their normal position. Near the x-organ, just under the connective tissue sheath covering the medulla terminalis of each eyestalk, considerable bluish-white secretory material had accumulated. Fixed and stained with chrome-hematoxylin-phloxin, similar secretory material from a crab, seven and one-half months after bilateral sinus gland removal, resembled histologically (Fig. 7) that found in a normal sinus gland. Fibers from neurosecretory cells of the eyestalk and brain could be traced into this regenerated sinus gland.

Twenty-two days after the x-organ nerve and part of the x-organ itself had been removed from each eyestalk of a crab, one eyestalk retained no sign and the other eyestalk only faint traces of a normal sinus gland. Fibers of remaining x-organ cells and axons from the optic lobe peduncle together had produced on the medulla terminalis next to the x-organ a regenerating sinus gland, part of which is sketched in Figure 8. Bulbous nerve endings and their acidophilic contents, in which were included basophilic granules, appeared in the same sort of inflorescence as is typical of the normal sinus gland (Fig. 4). Granules lined the nerve fibers and their swollen endings.

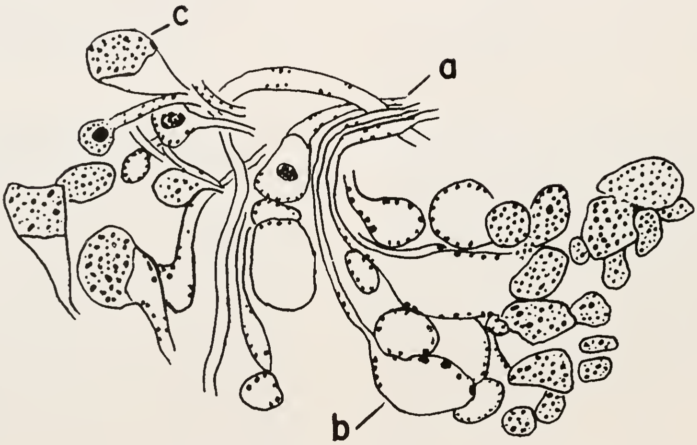

FIGURE 8. A portion of a regenerating sinus gland (right eyestalk of *Gecarcinus lateralis*) which appeared as illustrated after partial removal of both x-organs and x-organ nerves three weeks before had caused degeneration of the normal sinus glands. Secretory granules line the nerve fibers (a) and their swollen endings (b). Many granules have accumulated within masses of acidophilic secretory material (c) which lie at the tips of the bulbous nerve endings.

Removal of a normal sinus gland seems to cause partial degeneration of neurosecretory cell axons. Upon subsequent regeneration they reach the surface of the ganglion at a point close to the x-organ, where they form a structure in many ways resembling the original sinus gland. Certain respiratory data (Bliss, 1952) suggest that although regenerated sinus glands of *Gecarcinus* continue to release secretory material, the mechanism of release is not normal.

Of considerable interest are the recent reports that regenerated sinus glands are formed in several species of crabs not only after sinus gland removal but also after

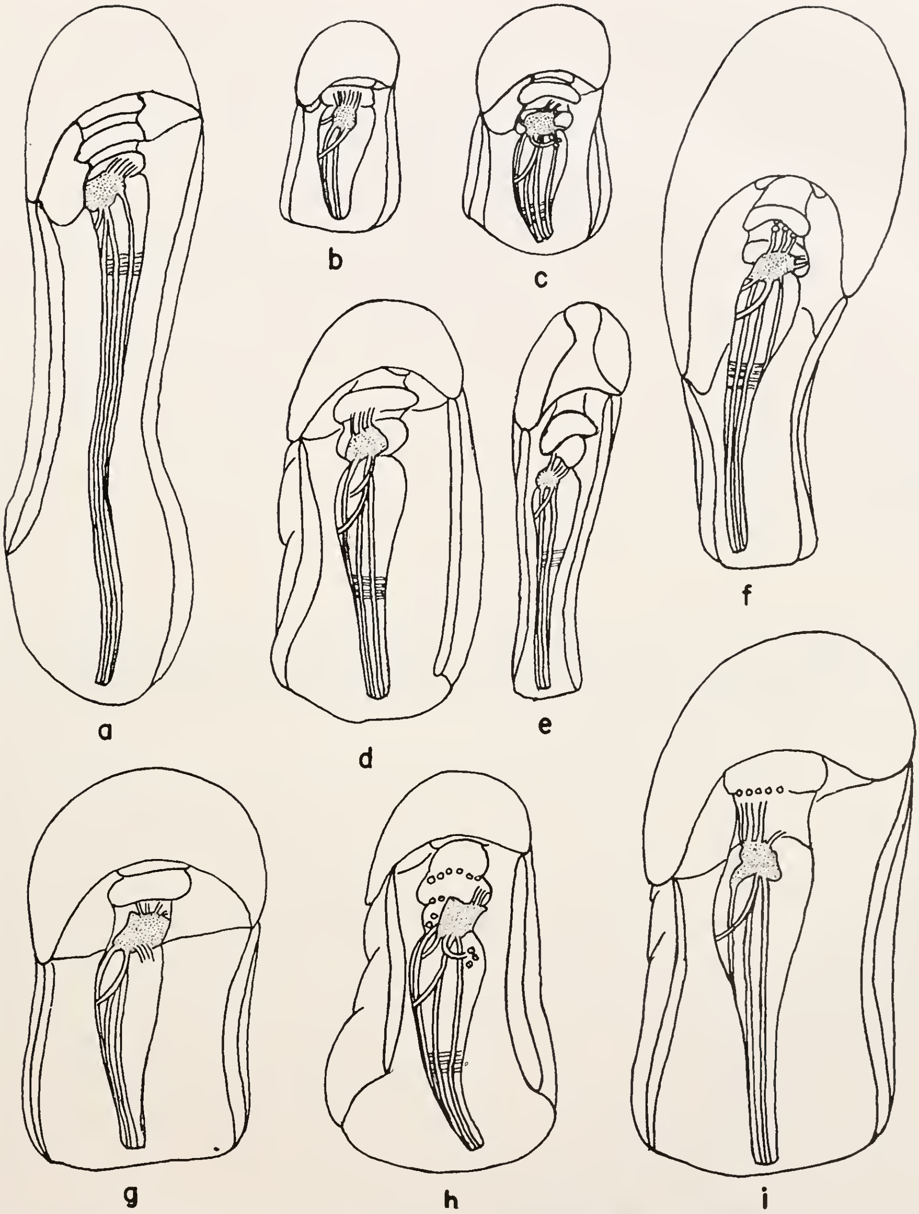

FIGURE 9. Right eyestalks of brachyurans. (a) *Cardiosoma guanhumi;* (b) *Pachygrapsus transversus;* (c) *Sesarma* (*Holometopus*) *ricordi;* (d) *Callinectes ornatus;* (e) *Uca mordax;* (f) *Ocypode albicans;* (g) *Portunus* (*Achelous*) *spinimanus;* (h) *Carcinides maenas;* (i) *Grapsus grapsus.* The eyestalks of *Callinectes exasperatus* are similar to those of *C. ornatus.* Stippled areas represent sinus glands. With one exception (*Uca mordax,* 3½ ×), all drawings are magnified approximately 2½ times as much as is the *Cardiosoma* drawing. Actual length of adult *Cardiosoma* eyestalk: 2–2½ centimeters.

transection of the nerve from the x-organ (Enami, personal communication; Passano, 1951a, 1952b).

The sinus glands of *Gecarcinus* are receiving centers and storage depots. For some unexplained reason, conceivably related to control of hormone release or perhaps to activation of inactive precursors, secretory products synthesized in the central nervous system are carried by nerve fibers to their swollen endings, the so-called sinus gland, before being released into the hemolymph.

Is this complex synthesizing-transporting-storing-releasing system unique to *Gecarcinus* or does it exist in other Brachyura and in Macrura as well? Observations on eyestalks (Fig. 9) and brain of ten additional species of crabs have revealed the same general pattern of bluish-white globules and nerve tracts and have affirmed the universality of this neurosecretory system in tail-less decapods. The eyestalk and brain morphology of *Cambarus,* to be presented in a later paper, demonstrates the existence of a corresponding system in tailed forms. It is now justifiable to extend the concept first proposed for vertebrates and insects (Scharrer and Scharrer, 1944) to another large group of invertebrates, the decapod Crustacea, and to underscore the possibility that basically similar neurosecretory mechanisms may exist throughout the animal kingdom.

Discussion

Ever since Hanström described the x-organ of the Decapoda, it has been sought and found in many species of Crustacea. Its secretory nature was recognized cytologically but no endocrine function could be attributed to it, particularly since portions of the medulla terminalis containing the x-organ were shown by Hanström to have no chromatophorotropic activity.

The sinus gland of Decapoda has been described by Hanström as a differentiation of the neurilemma (sheath), which had become thick, syncytial, full of radially arranged canals containing secretory material, and innervated by a large, coarse-fibered nerve from the medulla terminalis (Hanström, 1939). Subsequent cytological studies on the sinus gland resulted in confusion concerning its nature and structure. Dethier (1942) described the sinus gland of *Cambarus* as composed of anastomosing rows of cells, yet Pyle (1943) observed no cell boundaries in the sinus gland of *Pinnotheres* or *Homarus* and noted its distal migration during the larval development of *Pinnotheres*. He asked in effect: is the sinus gland a non-cellular storage syncytium?

Smith (1948) observed the bluish-white color of the living sinus gland nerve and the presence of an eosinophilic secretion product in the fixed preparation. A year later, from his experiments on retinal pigment migration following sinus gland removal, he concluded that production of retinal pigment activator in *Hemigrapsus* and *Pachygrapsus* occurs not only in the sinus gland but in many parts of the nervous system as well. For a second time the idea that the sinus gland might be specialized for storage was proposed, Smith suggesting in addition the possibility that the organ might facilitate periodic hormone release. Bowman (1949) found the results of chromatophore experiments in *Hemigrapsus* understandable when the highly active sinus gland was considered a storage-release organ for material produced by chromatophorotropically-active regions of the central nervous system.

The recent observations that eyestalk and x-organ removals, but not sinus gland removal, induce molting or physiological changes associated with molt (Bliss, 1951, 1952; Frost, Saloum and Kleinholz, 1951; Havel and Kleinholz, 1951; Passano, 1951b, 1952b; Travis, 1951a, 1951b; Welsh, 1951), coupled with microscopic evidence of the presence of secretory material along the nerve connecting x-organ (or beta cells) with the sinus gland (Bliss, 1951, 1952; Durand, personal communication; Enami, 1951; Passano, 1951a, 1952a, 1952b) and along nerves from other neurosecretory cell groups in the eyestalk and brain, have led to the hypothesis presented in this paper, namely, that a large neurosecretory system, involving many parts of the central nervous system, exists in decapod crustaceans, and that the sinus gland is primarily, and perhaps exclusively, a storage and release site for material produced within this neurosecretory system. The initial suggestions made by Pyle, Smith and Bowman have been shown to be justified.

Before concluding, the authors wish to make brief comment on a paper by Enami (1951). After a series of careful histological studies on the eyestalks, brain, and thoracic ganglia, in which the neurosecretory cells of three species of *Sesarma* were mapped, Enami has concluded that the sinus gland and the neurilemma of this organ carry on nuclear secretion. Nothing the present writers have seen has indicated such a process. They suggest that the bulbous nerve endings which make up the sinus gland may have been interpreted by Enami as nuclear capsules and the acidophilic material within those endings as nuclear secretion products.

If the organ which has been named the sinus gland releases but does not synthesize products of secretion, should it still be known by that name? It is an integral part of a diffuse neurosecretory complex, composed of cell bodies, axons, and their swollen endings. It is the portion of this complex which accomplishes the essential processes of storage, possibly of transformation, and of release.

What purposes are served by a diffuse neurosecretory system in contrast to a discrete compact gland? This question can be answered only as physiological data accumulate, but certain possibilities can be suggested. With a large part of the central nervous system occupied by secretory cells and their fiber tracts, it is likely that their specialization for neurosecretion has not eliminated their capacity to act as conductors of nerve impulses. A neurosecretory cell group could then trigger the release of its own secretory material, by conducting impulses to its endings in the storage site. If formed from completely independent groups of neurosecretory cells, the sinus gland might serve as the storage-release center for more than one hormone (see Brown, 1944, 1951; Brown, Sandeen and Webb, 1951; Brown and Hines, 1952; Scharrer, 1953).

SUMMARY

1. The land crab, *Gecarcinus lateralis* (Fréminville), was selected as the principal subject for this study of anatomical relationships existing between neurosecretory centers in the eyestalks and brain of brachyuran Crustacea. Observations were also made on ten other species of crabs.

2. Techniques useful in clarifying crustacean endocrine structures have been described.

3. It has been found that the sinus gland is actually a mass of swollen nerve fiber endings, arranged in the form of an inflorescence and bearing secretory

material. The histological structure of regenerated sinus glands, which appear after sinus gland removal, resembles that of normal sinus glands.

4. Nerve fibers, the endings of which compose the sinus gland, originate in neurosecretory cells of the brain, the eyestalk ganglia, and possibly the thoracic ganglionic mass. X-organ fibers contribute their endings to the sinus gland.

5. Neurosecretory products of the living preparation appear as bluish-white material in the cell bodies, nerve tracts, and sinus gland. When stained with Gomori's chrome-hematoxylin-phloxin, neurosecretory material assumes a homogeneous acidophilic or granular basophilic form.

6. There is developed the concept of a large neurosecretory system involving the brain, cells of the eyestalk ganglia, and perhaps those of the thoracic ganglion, all of which transmit their secretory products to storage-release organs, the so-called sinus glands.

7. The similarities between this system in decapod crustaceans, the hypothalamo-hypophyseal system in vertebrates, and the intercerebralis-cardiacum-allatum system in insects are emphasized. It seems justifiable to extend to the decapod crustaceans the concept first proposed for vertebrates and insects by Scharrer and Scharrer (1944).

8. Recent literature on the histology and physiology of the sinus gland and x-organ is reviewed and interpreted in terms of the hypothesis, proposed in this paper, of a crustacean neurosecretory system.

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EFFECTS OF X-RAYS UPON HAPLOID AND DIPLOID EMBRYOS OF HABROBRACON[1]

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Correlations between radiosensitivity and ploidy, the number of chromosome sets, have been made by Stadler (1929), Müntzing (1941), Fröier, Gelin and Gustafsson (1941) and Smith (1943, 1946) for cereals; by Latarjet and Ephrussi (1949) for the yeast, Saccaromyces; by Whiting and Bostian (1931), Clark and Kelly (1950), and Clark and Mitchell (1951) for Habrobracon; and by Lamy and Muller (1939) for Drosophila. These studies, with the exception of the last, have been consistent in demonstrating, for the material tested, that there is a greater resistance with higher ploidy and the results have been taken as evidence that x-radiation damage is primarily chromosomal. That there is less chance for homozygous deficiencies to occur in polyploids than in diploids and that all deficiencies in haploids would be lethal, have been given as explanations. Lamy and Muller found that diploid and triploid Drosophila x-rayed as embryos are equally radiosensitive and they assume that the deleterious effects in this case are largely non-chromosomal ("physiological").

The senior author and associates have been studying the effects of x-rays on haploids and diploids of the parasitic wasp, Habrobracon, during different stages of its life cycle in order to determine to what extent genome number can be correlated with radiosensitivity. Comparison of radiosensitivity during the pupal, prepupal and larval stages has shown that diploids are more resistant than haploids. The present paper reports on radiosensitivity during the early embryonic stages.

MATERIAL AND METHODS

In Habrobracon, haploid males arise from unfertilized eggs and diploids (male and female) from fertilized eggs. Whiting (1943) has established the sex-determining mechanism and has shown that cultures can be obtained that contain (1) only haploid males, (2) haploid males and diploid females, (3) haploid males, diploid females and diploid males. In the present study comparison is made between (1) and (2). Stocks No. 33 and No. 17-o^i (ivory) were used. Cultures of haploid embryos were obtained from No. 33 unmated mothers while cultures of haploid and diploid embryos ("mixed" cultures) were obtained from No. 33 females mated to No. 17-o^i males. Progeny of mated females normally consist of about 60 per cent diploids and 40 per cent haploids.

Embryos of known ages were x-rayed. They were then (a) placed in syracuse dishes containing a mineral oil ("Nujol") and observed for hatchability or (b)

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placed upon food (paralyzed Ephestia larvae) in order that survivors could develop into adults. All cultures were kept at 30° C. At this temperature eggs hatch about 30 hours after being laid. The larvae in mineral oil were counted 40 hours after oviposition and the incidence of hatchability noted. Eggs placed upon Ephestia larvae were observed at various stages of development and comparison of sex ratios was made between control and treated cultures from mated females.

All microscopical studies were made upon whole mounts of embryos fixed in Kahle's fluid and stained with the Rafalko modification of the Feulgen technique.

For x-ray experiments a dual-tube self-rectifying outfit with a simultaneous cross firing technique was used. The secondary voltage was 182 kv.; the tube was 25 ma.; the output intensity was 110 r per minute. There was an inherent filter equivalent to 0.2 mm. copper. X-radiation was obtained at the Marine Biological Laboratory, Woods Hole, Massachusetts.

RESULTS

Embryos treated between one and three hours of age

As determined by microscopical study of whole mounts, untreated embryos between one and three hours of age were found not to have progressed beyond cleavage. There is a syncytium at this stage with the nuclei rapidly undergoing synchronous mitosis. For the majority of the eggs the number of nuclei found was over fifty. A range from the pronuclear stage to embryonic stages having over 250 nuclei was observed but these extremes were exceptional. Eggs from mated and unmated females were in the same stages of development.

TABLE I

Hatchability ratios for eggs from mated and unmated females
(Age of embryos when x-rayed, 1-3 hours)

Dose (r)	Haploids (from unmated ♀ ♀)		Haploids	Diploids*	Diploids (estimated) hatchability
			(from mated ♀ ♀)		
	No. eggs	Hatchability	No. eggs	Hatchability	
0	573	.93±.01	1071	.92±.01	.91±.01
27.5	91	.97±.02	131	.93±.02	.90±.04
55	148	.91±.02	263	.87±.02	.75±.03
110	263	.85±.02	433	.47±.02	.22±.03
165	220	.69±.03	168	.31±.03	.07±.02
220	331	.51±.03	319	.18±.02	.00
275	137	.27±.04	146	.05±.02	
330	195	.10±.02	225	.04±.02	
440	142	.03±.01	161	.02±.01	

* Adult female ratio in controls .60 (729/1226 adults).

Hatchability ratios for eggs of this age at time of treatment demonstrate that those from mated females are more sensitive than those from unmated (Table I). This difference is clearly shown after doses from 110 r to 275 r. For example, after treatment with 110 r, the hatchability for eggs from mated females is 0.47 ± .02 as compared with 0.85 ± .02 for eggs from unmated mothers. Untreated eggs from mated and unmated females do not differ in hatchability (Table I). Since the incidence of female offspring from mated control mothers was found to be 0.60 on the basis of 1226 adults counted, these data can be used for deriving the hatchability of diploid eggs (Table I).

Since the hatchability data (Table I) indicate that haploids are more radioresistant, the ratio of adult females $\left(\frac{\text{♀♀}}{\text{Total}}\right)$ from x-rayed eggs of mated mothers should be lower than from control eggs. This is substantiated when comparison is made of the ratios of haploid males and diploid females from x-rayed and control cultures (Table II). At all doses there is a marked decrease in the ratio of females. For example, after treatment with 220 r, 33 males and 6 females were obtained (0.15 ± .06) as compared with 46 males and 60 females in the untreated group (0.57 ± .05).

Reference to Table II will show that there is a decrease in hatchability with increase in dose and that this occurs for eggs from both mated and unmated females.

TABLE II

Hatchability and eclosion ratios for offspring from mated females
(*Age of embryos when x-rayed, 1–3 hours*)

Dose (r)	No. Eggs	No. adults		$\frac{\text{Larvae}}{\text{Eggs}}$	$\frac{\text{Adults}}{\text{Larvae}}$	$\frac{\text{♀♀}}{\text{Total}}$
		♂♂	♀♀			
0	146	46	60	.84±.03	.86±.03	.57±.05
55	116	43	43	.81±.04	.91±.03	.50±.05
110	101	26	7	.55±.05	.59±.07	.21±.07
165	284	42	8	.18±.02	.98±.02	.16±.05
220	404	33	6	.11±.02	.86±.05	.15±.06

Once the eggs have hatched, however, there appear to be no further deleterious effects as shown by comparison of ratios of adults/larvae at different doses. There is no developmental lag in survivors and the adults show no structural abnormalities. Death due to irradiation occurs before hatching.

Embryos that were x-rayed (220 r) during cleavage (one–three hours of age) were fixed at an age when somites were normally present (12–15 hours of age) in the control embryos. Examination of whole mounts showed that some of these treated embryos had formed somites and appeared normal. The majority, however, were either in cleavage or early blastema. Nuclei in the cleavage stages were in interphase and were very much enlarged, being up to four times the diameter of untreated nuclei. Many were clumped together forming dark patches within the egg. The large number of nuclei in treated embryos which had died may suggest that cleavage continued after x-radiation. In some eggs, nuclei could not be found, due, perhaps, to disintegration of chromatin material. A sufficient number of eggs from haploid and mixed cultures was not prepared to make a quantitative microscopical comparison between these groups.

TABLE III

Hatchability and eclosion ratios for offspring from mated females
(Age of embryos when x-rayed, 3–4 hours)

Dose (r)	No. eggs	No. adults		Larvae/Eggs	Adults/Larvae	♀♀/Total
		♂♂	♀♀			
0	197	53	102	.89±.02	.89±.02	.66±.04
220	442	48	81	.34±.02	.86±.03	.63±.04

Embryos treated between three and four hours of age

Embryos of three–four hours of age from mated mothers were treated with 220 r and were allowed to develop into adults. The sex ratios of adults emerging were compared with those from control eggs (Table III). The incidence of females from control and treated eggs is not significantly different. This indicates that haploids and diploids are equally radiosensitive when treated at this stage of development. There are no developmental effects of an injurious nature after hatching as shown by the ratios of adults/larvae. Most of the three-hour old control embryos have completed cleavage and have hundreds of nuclei within the egg. These nuclei are migrating or have migrated to the periphery of the egg (blastema stage).

Embryos treated between 13 and 15 hours of age

Older embryos in the stage when somites are present that are treated with x-rays may not show injury during the egg stages. However, deleterious effects may be seen during later stages of development. Embryos, 13–15 hours of age, were x-rayed with either 722 r or 1444 r. Comparison of the number of larvae obtained showed that there is no decrease in hatchability over the controls (Table IV). The number of adults, however, is markedly decreased for those treated with 1444 r. These groups tended to show a lag in development as larvae and to remain as larvae after the controls had become adults. Some of the larvae did not grow to full size, but continued development to the pupal stage without spinning a cocoon.

TABLE IV

Hatchability and eclosion ratios for offspring from mated and unmated females
(Age of embryos when x-rayed, 13–15 hours)

Dose (r)	Mothers	No. eggs	No. larvae	No. adults		No. dying as larvae	Larvae/Eggs	Adults/Larvae
				♂♂	♀♀			
0	mated	42	30	11	12	2	.71	.77
722	mated	80	64	27	20	8	.80	.73
1444	mated	53	40	4	3	25	.75	.17
0	unmated	42	39	35		4	.93	.90
722	unmated	47	43	36		7	.92	.84
1444	unmated	50	44	12		24	.88	.27

There are insufficient data to state whether haploids and diploids are affected differentially when irradiated at 13–15 hours of age.

Discussion

Comparisons of radiosensitivity among individuals differing in ploidy have appeared in the literature in an attempt to evaluate the extent of chromosomal ("genetic") and cytoplasmic ("physiological") injury. Since differences in the number of chromosome sets are the most obvious distinguishing characteristics, the occurrence of a differential lethal effect has been taken as a criterion that nuclear injury has taken place. It is less obvious, but pertinent, that different metabolic conditions may exist in diploids and polyploids. Investigations herein reported of x-ray effects for haploids and diploids of Habrobracon suggest that radiosensitivity cannot be correlated with genome number at all stages in the life cycle. The differential radiosensitivity between haploids and diploids depends upon the stage of development at which they are irradiated. Haploids are more resistant than diploids during cleavage, equally resistant immediately following cleavage, and less resistant than diploids during the larval, prepupal, and pupal stages. One might inquire, therefore, if a differential effect between diploids and polyploids is an adequate criterion for distinguishing between chromosomal and cytoplasmic injury.

Most of the investigators who have reported a differential effect upon survival have found that the individuals with the greater number of chromosome sets are more resistant. Some of them (Müntzing, 1941; Latarjet and Ephrussi, 1949; Clark and Kelly, 1950; Clark and Mitchell, 1951) have suggested that (1) chromosome breakage occurs with the resultant loss of acentric fragments, and (2) there is a greater chance for individuals with higher ploidy to retain unbroken chromosomes that could compensate for the homologous fragments that are lost.

There are some observations that are difficult to reconcile with interpretations of radiation injury purely in terms of a chromosome breakage hypothesis. Studies on diploid and polyploid cereals have correlated gross observations on survival with the number of chromosome breaks (Fröier, Gelin and Gustafsson, 1941; Smith, 1943, 1946). These investigators have shown that polyploids, even though more resistant than diploids, have more anaphase bridges and chromosome fragments than comparable diploids. Fröier, Gelin and Gustafsson (1941) have shown that the germination and sprouting ability of polyploids were unimpaired even when 50 per cent of the mitoses had bridges and fragments, but the growth of the diploids was impaired with much less nuclear disturbance. Further, they showed that at high doses (50,000–60,000 r) nuclei of *Avena sativa,* a hexaploid, are still able to divide even if the chromosomes are fragmented to the extent of discontinuity. Marshak and Bradley (1944) have shown that mitotic inhibition is inversely proportional to the number of chromosomes, but independent of chromosome length. They suggest that this indicates an effect upon the centromeres.

If a chromosomal basis for injury is to be postulated, then a different mechanism must be used to explain the fact that haploid embryos of Habrobracon are more resistant than diploids when treated during cleavage. The observations by Fröier, Gelin and Gustafsson (1941) and Smith (1943, 1946) on cereals and by Bishop (1950) on Tradescantia, that the number of chromosome breaks following x-radiation is proportional to the number of chromosomes, would suggest that the diploid nuclei of Habrobracon incur twice as many chromosome breaks as comparable haploid nuclei. One might reason that the diploids would be more sensitive than the haploids because they have received twice as much injury per nucleus. It has already been noted, however, that polyploids with more chromosome breaks per nucleus are more resistant than diploids with fewer chromosome breaks per nucleus. Differences between the stages of development that allow haploids to be more resistant at one stage and more sensitive at another should perhaps be considered. A number of obvious differences exist between the cleavage stage and the later post-embryonic stages of Habrobracon. During cleavage there is a syncytium with the nuclei dividing rapidly and synchronously throughout the embryo while in later embryonic and the post-embryonic stages cells are present with active proliferation being restricted to localized regions. There is little differentiation in the embryos during cleavage. Whether or not these differences have anything to do with the reversal in radiosensitivity is not known.

If differential radiosensitivity is to be taken as a criterion of chromosomal injury, then the equivalent radiosensitivity of haploid and diploid embryos when x-rayed immediately after cleavage must be taken to mean that extra-chromosomal factors are involved. The radioresistance is increasing rapidly at this time and, therefore, a slight lag in development between haploids and diploids could result in a large difference in radiosensitivity. Kelly (unpublished), using 50 per cent hatchability as the criterion for comparative lethality, has shown that haploid embryos increase in resistance from a dose of about 200 r during cleavage to 7000 r in about four hours. Lamy and Muller (1939) found that diploid and triploid Drosophila when treated as embryos were equally radiosensitive. They explained this by assuming that death was due to a "non-genetic" ("physiological") type of injury. The data reported in the present paper suggest that the Drosophila embryos were x-rayed after cleavage.

Embryos x-rayed during cleavage or in early blastema are arrested in development during the egg state or not at all. Post-embryonic development is normal. Embryos x-rayed at a later stage, however, may show a post-embryonic lag in development and become arrested at the larval stage. Dent and Amy (1950) treated embryos and larvae of Habrobracon with P^{32}. They found a lag and an arresting of development at the larval stage. Henshaw and Henshaw (1933) and Packard (1935) have determined the sensitivity of Drosophila eggs when x-rayed during different stages of embryonic development using hatchability as the criterion of radiosensitivity. Since in Habrobracon older embryos when x-rayed may hatch normally but may show deleterious effects after hatching, it seems that hatchability alone is not an adequate measure of radiosensitivity. Kelly (unpublished) showed that an x-ray dose of 30,000 r had no effect on the hatchability of older embryos of Habrobracon. Such embryos, however, would not have developed beyond the larval stage.

Although its significance is not known, the observation that nuclei of embryos arrested during cleavage are considerably larger following irradiation is in agreement with the reports of enlarged nuclei by Mottram (1933) for Colpidium, Jenson's rat sarcoma and bean roots, and by Harrington and Koza (1951) for grasshopper embryos.

These data on Habrobracon show that radiosensitivity cannot be correlated with genome number throughout the life cycle. It is difficult, therefore, to pose a single generalization that will explain these diverse data from the standpoint of chromosomal injury. It may also be difficult to explain them on the basis of a quantitative difference of some chemical constituent. It is possible that there is no single "most-radiosensitive" mechanism, but that the relative sensitivities of the cellular materials change during development and that different mechanisms may be primarily involved at different stages.

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SUMMARY

1. Comparison of radiosensitivity of haploids and diploids of Habrobracon shows that the stage of development at which the animals are x-rayed is important in determining the relative sensitivity between these groups. When embryos are x-rayed during cleavage, haploids are more resistant than diploids; when embryos are x-rayed immediately after cleavage has been completed (blastema stage), haploids and diploids are equally radiosensitive.

2. Embryos x-rayed during cleavage or early blastema are deleteriously affected during the egg stage or not at all. Those that hatch complete post-embryonic development normally. Older embryos when x-rayed may hatch, but post-embryonic development is slowed down and many of the individuals are arrested in development as larvae. Hatchability, therefore, is not an adequate criterion of radiosensitivity for older embryos.

3. Embryos that are x-rayed during cleavage and fail to hatch are arrested in cleavage or in early blastema. The nuclei are arrested at interphase and become enlarged up to four times the diameter of untreated nuclei.

4. Since the differential radiosensitivity between haploids and diploids depends upon the stage of development at which they are irradiated, it is difficult to pose a single hypothesis that will account for these facts. It seems reasonable to consider that the relative sensitivities of the cellular materials change during development and that different mechanisms may be primarily involved at different stages.

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ADAPTATION TO CHEMICAL STIMULATION OF THE TARSAL RECEPTORS OF THE BLOWFLY[1]

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In the course of several decades of studies of chemoreception in insects the existence of some demonstrable form of adaptation was speculated upon by many workers. Some actually designed specific experiments to develop the idea. These experiments usually took the form of a comparison of a taste threshold measured by means of a descending series of concentrations with a threshold obtained by ascending presentation. Among the investigators who made the comparison, Marshall (1935) and Eger (1937) could demonstrate no difference; Verlaine (1927), Weis (1930) and Kunze (1933) believed that such differences existed, but their data in support of the belief are statistically poor; von Frisch (1934) could detect no change in threshold but did observe modifications of behavior which suggested that adaptation did occur; Minnich (1929) observed clear-cut differences in threshold. Both during and since that period extensive use was made of a convenient reflex of flies and butterflies, the proboscis response; but the data which the method yielded in the hands of the pioneer workers were contradictory and of questionable significance. Now, as a consequence of recent advances in the field, fuller advantage may be taken of this easily observed and decisive response to investigate profitably the phenomenon of adaptation and to reconcile the divergent older results.

When receptors located on the tarsi are stimulated by solutions of certain sugars, among them being sucrose, glucose and fructose, the insect responds by extending its proboscis and, when permitted, by drinking. Upon cessation of stimulation or initiation of stimulation by a wide variety of non-saccharide materials (*e.g.*, electrolytes and many organic compounds) the proboscis, if extended, is retracted. If, prior to stimulation by unacceptable compounds, the proboscis is already in the retracted position, there is no discernible response involving the mouthparts. In order to study the stimulating effect of unacceptable compounds it has been the custom to mix different amounts of the material in question with sugar solutions which are then presented to the insects. In this manner one is able to ascertain the minimum concentration of an unacceptable compound which will prevent a response to sugar. Thus, the two basic techniques employed in all experiments on contact chemoreception involve the determination of acceptance thresholds and of rejection thresholds.

Any of three standard operational procedures may be followed in obtaining threshold data: (1) each insect of a sample population of two hundred or more is stimulated by each concentration in turn presented in ascending order until a

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[1] The work described in this paper was done under contract between the Medical Division, Chemical Corps, United States Army, and The Johns Hopkins University. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the author.

response is obtained; (2) each fly is stimulated by each concentration in turn presented in descending order until an appropriate response is elicited; (3) the insects are random-sampled, *i.e.,* a different group is tested at each concentration and note taken of the percentage of each class which responds. Median values for the threshold may be developed from any of these procedures by the proper mathematical treatment (see Dethier and Chadwick, 1948). As will be shown below, use of these procedures on a comparative basis affords excellent opportunities for investigating the phenomenon of adaptation.

I. DEMONSTRATION OF ADAPTATION

Methods and results

Random Series. Several hundred one to three day old flies (*Phormia regina* Meigen) from a standard culture were mounted on sticks according to the method described by Dethier and Chadwick (1947). Just prior to testing, each fly was given water *ad libitum.* A series of sucrose solutions of doubling steps of concentration from 0.0005 M to 1 M was prepared. The flies were divided into as many groups of twenty each as there were concentrations of sucrose. Each group was tested at a different concentration. A test consisted of lowering the legs of each fly first into water until no proboscis extension was elicited and then placing the legs into the test solution for two seconds. The percentages of each group responding at each concentration were converted to probability units and plotted against the logarithms of the respective concentrations. The most probable value of the log concentration accepted by 50% of the flies was then determined according to the method of Bliss (1938).

Ascending Series. The same series of solutions as before was prepared. Each fly was tested as follows: (1) its legs were placed in water, and if a response was elicited, the animal was allowed to drink its fill; (2) when the response to water was negative, the fly was placed in 0.0005 M sucrose for two seconds; (3) if no response was forthcoming, the fly was transferred directly to the next higher concentration for two seconds and similarly on up the concentration series until a response was elicited. In no test was the proboscis ever allowed to touch the test solution. Once a fly had responded to a given concentration, it almost invariably responded to all higher concentrations. As soon as all flies had been tested the raw data were sampled in the following manner. A table with as many columns as there were concentrations was constructed. The specimens were now sampled, five at a time, in the order in which they had appeared for testing. For the first group of five the number accepting at the highest concentration was recorded in the table; for the next group, the number accepting at the next lower concentration; and so on in rotation until all of the flies used in the test had been recorded. The percentages accepting at each concentration were calculated from the totals in the columns and treated as above.

In an ascending series the responses of the population usually extended over ten doubling concentrations. Consequently, it was the custom to test a minimum of two hundred flies so that the n for each class was never less than twenty.

Descending Series. The method of running descending series differs from that of ascending series in the following respects. After being tested in water flies are presented first with a high concentration, *e.g.,* 1 M, known, as a result of information derived from random series, to be above threshold. After two seconds each fly is then presented directly with the next lower concentration and so on down the series until the proboscis is withdrawn. When this occurs, the animal is retested against the starting concentration as a check against absolute refractoriness. If he continues down to the lowest concentration in the series with no sign of cessation of response, he is retested in water. Such a fly invariably drinks greedily, and the test is discarded. Not infrequently flies which are negative to water at the beginning of a run develop a desire for water in the course of the run. Data are accumulated and manipulated as already described. No significant difference in threshold is obtained if the feet are washed in water between successive concentrations provided the time consumed does not exceed a few seconds.

It is important to keep in mind the fact that descending series may be started at any supraliminal concentration.

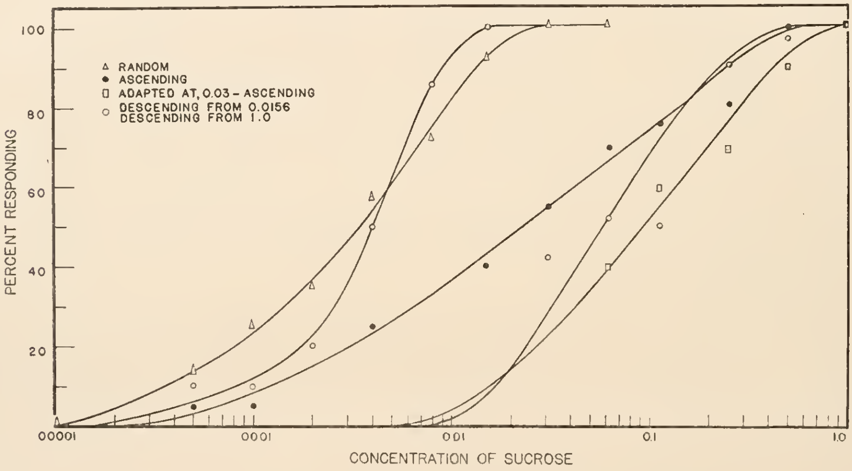

FIGURE 1. Change in the acceptance threshold of the blowfly to sucrose brought about by altering the order of presentation of different concentrations.

Adapted at One Concentration. It was feasible to start these series at any concentration, either subliminal or supraliminal. If, for example, a supraliminal concentration such as 0.03125 *M* was chosen as the starting point, each fly in turn was held with its feet in the solution until extension of the proboscis finally ceased. The animal was then run up the series in the usual manner until a fresh proboscis response was elicited. Again the data were random-sampled and treated as in the case of a normal ascending series. An alternative method was to random sample after adaptation. This procedure was carried out by dividing the flies into groups of twenty. Each fly in turn in group 1 was adapted, then tested at the next doubling concentration. The per cent responding was recorded. The members of group 2 were similarly adapted, then tested each in turn at the second highest doubling concentration and so on. If a subliminal concentration was chosen for adaptation, the feet were held in contact with the solution for ten seconds after which one of the procedures described above was followed.

Altogether, seven acceptance thresholds were determined by means of the techniques just described (see Section III): random sampled, ascending, descending from 1.0 *M*, descending from 0.0156 *M*, ascending after adaptation at 0.03125 *M*, random after adaptation at 0.0156 *M*, and random sampled after adaptation at 0.001 *M* (see Section III). Representative curves describing the distribution of these thresholds are seen in Figure 1. Statistical information relating to the median threshold values, standard errors, slopes and variance is given in Table I.

Discussion

It is clear from an inspection of Figure 1 that several different values for the acceptance threshold of sucrose by a given population of flies can be obtained and that the magnitude of these values depends upon the order in which stimuli are presented. It can also be shown (Section III) that time relations in the presentation of stimuli are of considerable importance. The data demonstrate that response to stimulation of the tarsi by sucrose is profoundly modified by previous stimulation. This effect of stimulation exhibits all of the characteristics of adaptation.

TABLE I

Acceptance and rejection thresholds of Phormia regina as ascertained from different methods of presentation of stimuli

Method of presentation	Median acceptance threshold	Log molar concentration accepted by 50%	$a \pm$S.E.	$b \pm$S.E.	$\bar{x}$	No. of flies tested
Random	0.00356	−2.449±0.0332	4.983±0.0531	1.596±0.117	−2.555	120
Ascending	0.023	−1.632±0.0966	4.972±0.0992	1.028±0.123	−1.659	220
Descending						
From 0.0156 *M*	0.00334	−2.476±0.0598	5.016±0.136	2.285±0.310	−2.469	120
From 1.0 *M*	0.0474	−1.324±0.0739	5.450±0.119	1.834±0.268	−1.079	240
Adapted at 0.03125—then ascending	0.0953	−1.021±0.0999	5.510±0.146	1.810±0.381	−0.739	100
Adapted at 0.001 random 1 sec.	0.0116	−1.935±0.0477	5.188±0.104	2.242±0.271	−2.019	250
4 sec.	0.0156	−1.807±0.0472	4.901±0.176	3.753±0.630	−1.933	100
10 sec.	0.0168	−1.776±0.0050	4.891±0.0897	1.802±0.234	−1.782	250
30 sec.	0.0114	−1.942±0.0439	5.041±0.173	3.939±0.613	−1.932	80
Adapted at 0.0156 random 12 sec.	0.0458	−1.339±0.0472	5.058±0.158	3.356±0.464	−1.322	100
30 sec.	0.0676	−1.170±0.0505	4.996±0.146	2.895±0.392	−1.171	100
Ethanediol in fructose						
Random	8.128	0.910±0.018	5.281±0.137	7.647±1.059	0.946	120
Ascending	8.241	0.916±0.024	5.220±0.144	6.182±0.900	0.951	160
Fructose in ethanediol						
Random	0.142	−0.849±0.096	5.093±0.122	1.276±0.251	−0.777	120
Ascending	0.142	−0.848±0.066	5.380±0.150	2.364±0.414	−0.730	100

The curve describing the distribution of thresholds of a sample of flies which has been random-sampled can be taken as the baseline of sensitivity for this laboratory population at a given age and level of nutrition and water balance. To this baseline all other thresholds will be referred, because its median represents the lowest concentration of sucrose which will elicit a response from 50% of the flies, a concentration which is effective if there has been no immediate previous stimulation. This basal value is 0.00356 *M*.

When successive concentrations of sucrose are presented in ascending order beginning at a concentration subliminal with respect to the random curve, the threshold is increased by a factor of approximately seven. In other words, individuals now fail to respond to solutions which are known to be capable of evoking a response if presented directly after water and in the absence of previous stimulation. Quite obviously the process of gradually increasing the stimulus by small increments from a subliminal value has caused a decrease of sensitivity to occur, so that a higher-than-normal concentration is finally required to trigger response. If this is so, it should be possible to effect a similar condition by prolonged exposure to a single selected concentration. This can be accomplished, as explained in the foregoing section, by placing the feet in a concentration which normally produces a response, waiting until the animal ceases responding, and then stimulating successively in an ascending series until the animal again responds. Similarly one can, after cessation of initial response, determine the final threshold randomly instead of successively. In any event the final threshold obtained depends upon the strength of the initial adapting solution. In the two cases tested the threshold is a concentration approximately three times that of the adapting concentration. Thus, it may be seen that the median value following adaptation at 0.0156 *M* is 0.04 *M* and following 0.03125 *M* it is 0.095 *M*. If adaptation is accomplished at a still higher concentration, the final threshold will be correspondingly higher.

Again, if the effects produced by previous stimulation partake of the nature of adaptation, one would logically expect that successive stimuli presented in decreasing order of concentration would yield a threshold value differing from those obtained with the foregoing methods. Further inspection of Figure 1 will show that this is indeed the case and that once again the value finally obtained depends upon the starting point of descent. When the initial stimulus is that which elicits approximately 100% response in the random series, *i.e.*, 0.0156 *M*, the final threshold does not differ greatly from the random series value. It is 0.00334 *M*. When the descent starts with 1 *M*, that which elicits 100% response from animals in an ascending series, the final threshold value (0.0474 *M*) is only slightly higher than the ascending value (0.023 *M*) but is many times higher than that of the other descending series. In other words, the final threshold value is determined by the point of departure.

These results naturally raise several critical questions. Can they be reconciled with the data of earlier workers? What are the respective contributions of the peripheral and the central nervous systems to the phenomenon? What is the nature of the process?

The failure of the majority of early workers to demonstrate clearly a difference in threshold with different techniques of presentation of stimuli, commonly ascending versus descending series, is due principally to the fact that such a long time interval was allowed to elapse between presentation of successive concentrations that recovery was essentially complete, and the effects of previous stimulation had been largely dissipated. Even under optimum conditions of presentation of stimuli the difference between ascending and descending threshold values is hardly demonstrable if identical ranges of concentrations are employed in the test. Marshall (1935) and Eger (1937), working with honeybees and caterpillars, respectively, routinely allowed 15- to 20-minute intervals between stimuli; consequently, it is not surprising that no threshold differences were demonstrated. The differences that Weis (1930), Verlaine (1927) and Kunze (1933), working with *Pyrameis atalanta, Pieris rapae* and the honeybee, respectively, reported are not statistically significant. Again, failure to show pronounced differences may be laid to the selection of time intervals. Weis, for example, presented ascending series essentially as described in this study, but in her descending series interpolated a 15- to 20-minute rest period between successive stimuli. Moreover, as shown above, it is not to be expected that there would be a great difference between an ascending series and a descending one when the latter begins at the top concentration of the former. Minnich (1929), on the other hand, studying the responses of *Calliphora vomitoria,* was able to arrive at a clear-cut difference because he compared the threshold obtained from an ascending series run as in this study with a threshold obtained by an ascending series in which the intervals between stimuli were so long (15 minutes) that the series was tantamount to being random. A period of 15 minutes appeared ample for complete recovery.

Thus it may be concluded that the contradictions in the published reports are not real, that the data are actually in agreement, and that previous stimulation does indeed affect the sensitivity of the system at one or more points.

II. Site of Adaptation

Methods

In order to determine whether or not failure of response to threshold amounts of sucrose following previous stimulation results primarily from a change in sensitivity of the receptors themselves or to adaptation at some distance from the peripheral system, that is, at a site in the central nervous system, an attempt was made to devise a method for separating the two effects. This was accomplished in part by adapting one leg and then testing the sensitivity of the contralateral leg. Several techniques were tested before two were decided upon. The first consisted of mounting each fly with all legs fastened to the wax block except the prothoracic pair and then tying a piece of fine silk thread to one of the free legs. This leg could now be raised or lowered into the test solution at will. Each fly was run up an ascending series of solutions in the usual manner except that the fettered leg was held out of the solution. As soon as a threshold of response had been obtained with the free leg, the unexposed leg, together with the adapted leg, was placed in a solution which was less concentrated but which was known from random series to be above the threshold of sensitivity. Forty-three flies were tested by means of this exacting technique.

The second technique made use of a partitioned dish which contained 0.125 *M* sucrose in one compartment and water in the other. A fly with only the prothoracic legs free was made to straddle the partition. The labellum was removed to prevent drinking. A proboscis response followed as a consequence of stimulation of one leg by the sucrose. As soon as the fly had become completely adapted, *i.e.,* retracted the proboscis and ceased responding, the legs were thoroughly rinsed in water, the fly was returned to the partitioned dish as before to insure that it was indeed adapted, it was again rinsed in water, and finally faced in the opposite direction and placed in the dish so that the adapted leg was now in the water compartment and the unexposed leg in the sucrose compartment. Thirty-six flies were tested.

Results and discussion

In every case in which hobbled flies were exposed successively to an ascending series of concentrations (first method), the fly afterwards failed to respond to a concentration below the ascending threshold when the unexposed contralateral leg was stimulated. A response could be elicited only by stimulating with a supraliminal concentration. Clearly, then, in this experiment the animal had become refractory in that it failed to respond even when a peripherally unadapted leg was stimulated. This behavior suggests that adaptation is central, but the possibility remains that the unnaturalness of the fettered condition rather than chemical adaptation may have been the limiting factor. However, the results were identical regardless of whether the free or the fettered leg was adapted.

On the other hand, when the divided dish was employed for testing, 66.6% of the flies which had been adapted on one leg responded vigorously when the contralateral leg was stimulated. Three major interpretations present themselves: (1) sucrose has not been washed from the stimulated leg completely, as a result of which the test with the contralateral leg actually amounted to bilateral stimulation; (2) adaptation is indeed peripheral; (3) adaptation occurs at a center in the CNS where there is no pooling of the contributions from both sides of the body.

With regard to the first possibility it can only be said that experience from many other sources would argue in favor of the rinsing being complete. If rinsing has not been adequate, the fact that addition of stimulation from the other leg provokes a response is an indication that the adapted leg is still contributing to the CNS and at least is not fully adapted; hence, the initial cessation of response results from CNS adaptation. On the other hand, if rinsing has been thorough, the return of response when the contralateral leg is stimulated indicates either that the first leg has been completely adapted or that the center into which it leads has been adapted. In either case stimulation of the fresh leg would activate a different site in the CNS and the animal would respond. The possibility of solving this problem appears remote. It must be added that not one of the 33.3% which failed to respond would respond (after adaptation) when both legs were placed simultaneously in 0.125 *M* sucrose. A greater concentration did provoke a response. Clearly these cases are indicative of a change in the central nervous system.

While it is surely unlikely that there is no adaptation in the receptors themselves, the more acceptable conclusion, all facts considered, is that the process which is being measured represents predominantly a state which has been brought about at some level in the central nervous system. If this be so, the condition may be analogous to the non-sensory adaptation in the olfactory system of man (Adrian, 1950) or to the phenomenon of inattention in general. It is indeed unfortunate that the tarsal system has not yet proved vulnerable to attack by bioelectrical techniques.

III. Time Course of Adaptation

Methods

Three different experiments were designed as a means of supplying information necessary for the plotting of adaptation curves. The first two conform more or less closely to the classical method of securing data for adaptation curves. Flies were adapted for varying periods of time to a selected concentration and the threshold measured after each period of adaptation. The experiment was repeated for several adapting concentrations. In the first method the adapting concentration was above the threshold of response (supraliminal). The actual method of procedure involved placing each fly of a sample of twenty in the adapting solution until the proboscis was retracted. This event was taken to mark zero time. The fly remained in contact with the solution for one second after zero time, after which it was exposed to a higher concentration for two seconds and the presence or absence of a response noted. Each remaining fly of the sample was treated similarly. Now another sample of twenty flies was placed one at a time in the same adapting solution also for one second and then tested at higher solution number two; a third sample was tested at higher solution number three and so on until five different samples of flies had each been adapted at the same concentration for an identical period of time and then tested for response at five different high concentrations. The per cent responding at each concentration was then calculated and from these values was determined the acceptance threshold characteristic of flies which had been adapted at a given solution for one second. The entire procedure was repeated at the same adapting solution for a period of two seconds, three seconds, and so on until a sufficient number of data had been obtained to plot a curve for that particular adapting concentration. The experiment was repeated in its entirety for another adapting concentration.

Method number two differed from the foregoing in one respect only. The adapting solutions were subliminal. Since there was no proboscis response to these adapting solutions, zero time was taken as the time at which the tarsi first came into contact with the solution.

Method number three consisted simply of ascertaining the time required for complete adaptation at each of several different concentrations. Data were obtained for each concentration by placing the feet of a fly in the solution and recording the interval of time which elapsed between extension of the proboscis, which occurred almost immediately after the feet touched the solution, and retraction. One hundred flies were tested at each concentration. It should be emphasized that the mouthparts were at no time in contact with the solutions.

The results of these three experiments are summarized in Table I. Data obtained by method number three are plotted in Figure 2.

Discussion

The fact that the rise in threshold following adaptation to supraliminal concentrations (method one) is independent of the duration of stimulation, once the proboscis has been retracted, and is constant, suggests that the change in sensitivity has attained completion at the time of retraction. The magnitude of the completed change varies as the adapting concentration, as a comparison of thresholds after continued exposure to different adapting concentrations has shown (Section I), but in each case it is complete. It follows from this that the development of adaptation must be studied at subliminal concentrations. When this is done (method 2) at 0.001 *M* for example, there are indications of an increase in threshold as the duration of continued stimulation increases. Thus, at zero time (no previous exposure) the threshold is 0.00356 *M;* at one second it is 0.0116 *M*, at 10 seconds it is 0.0168 *M*. No further increase can be demonstrated up to and including 30 seconds. From these data it would appear that adaptation of the type being measured at 0.001 *M* is complete after one second. Increases from one to ten seconds, while progressing in the correct direction, are not statistically significant. Since it is manifestly impossible with the preparation at hand to investigate sensitivity changes occurring in fractions of a second, further indications as to the shape

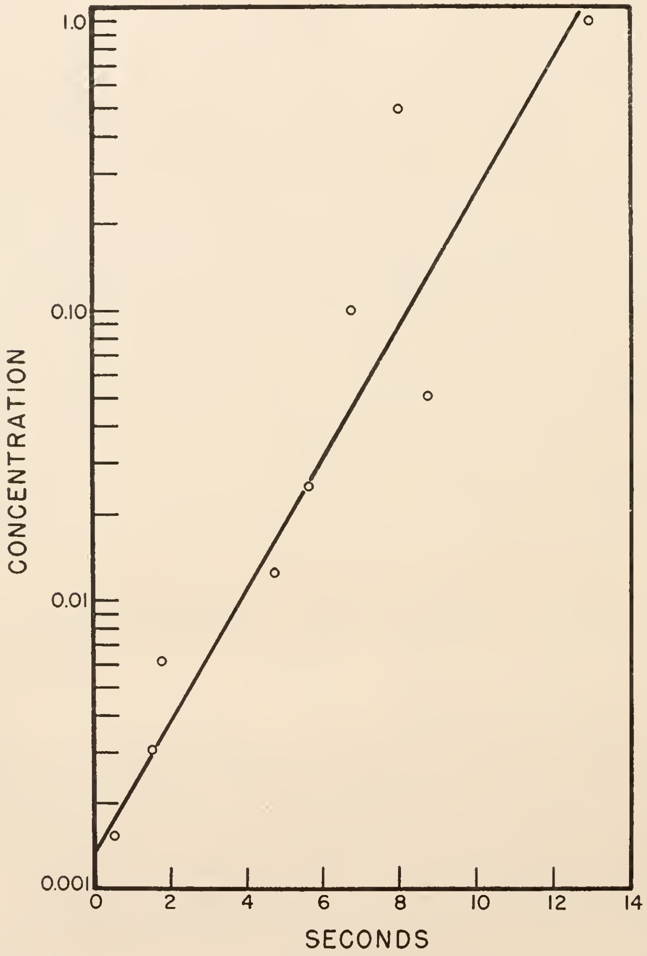

FIGURE 2. The relation between the concentration of sucrose employed as a stimulus and the time required for the cessation of response.

of the curve of adaptation cannot be obtained. On the other hand, it is possible experimentally to ascertain the time necessary for complete adaptation at different concentrations (method 3).

As an examination of Figure 2 shows, the time for complete adaptation as measured by time to retraction of the proboscis increases as the logarithm of adapting concentration. The increase is from about 1.5 seconds at 0.003125 *M* to about 13 seconds at 1.0 *M*. These data are in agreement with those from methods one and two in that method one indicates adaptation to be complete when the proboscis is retracted and method two indicates that at 0.001 *M* adaptation is complete after one second or less. A less complete set of data obtained with fructose in ethylene glycol is in substantial agreement and shows an increase in adaptation time from about 4 seconds at 0.006 *M* to 7 seconds at 1.0 *M*.

Even though it has not been possible to construct adaptation curves for *Phormia*, the fact that a correlation with adapting concentration can be demonstrated permits one to compare certain aspects of the phenomenon in *Phormia* and man. Hahn (1933) and Hahn, Kuckulies and Bissar (1940) have measured the course of adaptation of a variety of substances at different concentrations. The forms of the curves differ considerably, but two facts are worthy of mention: adaptation is complete in anywhere from 10 to 30 seconds; the time required for total adaptation increases as the concentration is increased. The situation in *Phormia* is remarkably similar. Adaptation times vary from one to 13 seconds; the total time required increases as the concentration is increased. Over the range of lower concentrations, 0.003125 *M* to 0.05 *M*, the time required is proportional to the logarithm of molar concentration. From 0.05 *M* on there is no significant change in time with increasing concentration.

Continuous stimulation at constant intensity for long periods of time results in bursts of activity. For example, a fly with its feet exposed to 0.05 *M* sucrose will continue to extend its proboscis for a period of 13 seconds on the average. Thereupon, the proboscis is retracted. After a varying period of time extension recommences; then it again ceases. Such rhythmic activity has been observed over periods of continuous stimulation lasting as long as three hours. Thus far no correlation between frequency of activity cycles and concentration has been observed.

IV. Simultaneous Stimulation by Mixtures of Solutions

No experiments were designed specifically to demonstrate the presence or absence of adaptation to compounds other than sugars, but work done in another connection throws some light on this problem.

Thresholds measured by the random technique and in ascending series were compared for each of two different mixtures, fructose in glycol, and glycol in fructose. In other words, determinations were made of the acceptance threshold of fructose in glycol and of the rejection threshold of glycol in 0.05 *M* fructose. The pertinent data are summarized in Table I.

In the case of each mixture there is no difference in the threshold value obtained by the random and the ascending methods. The most logical explanation of this fact is that adaptation occurs to each component of the mixture and that the processes balance each other. Otherwise, were there to be adaptation to fructose alone, the acceptance threshold should be higher in the ascending series.

The acceptance threshold would be lower in the ascending series if there were adaptation to the glycol alone. Similarly, were there adaptation to fructose alone, the rejection threshold to glycol would be lower in the ascending series. Since adaptation to fructose in glycol most certainly does occur, as was shown in Section III, it must be concluded that in mixtures of the sort described either adaptation to the two components progresses apace or that cross adaptation occurs. This last is highly unlikely.

Conclusion

The universality of the phenomenon of adaptation, in nervous tissue especially, would have led one to infer that tarsal chemoreceptors in blowflies could hardly act otherwise. The chief problem heretofore had been to demonstrate the existence of the process. Once demonstrated the problem remained of separating the sensory and central components. Unfortunately, realization of this hope has been but partial. The present data indicate, as would also have been expected, that the process as measured by behavior represents a combination of both components. Indications are that the central process proceeds to completion more rapidly and that it is the one which is manifested in the majority of experiments.

A more ambitious hope lay in the expectation that knowledge of the time course characteristics of the process might lead to clues to the nature of the process of chemoreception. This has so far been a barren hope, not only in the field of insect physiology but in the study of the human sense of taste as well. More fruitful comparisons do exist. The times required for total adaptation in the two species are of the same order of magnitude although in man the process which is measured is almost certainly peripheral; the time to total adaptation increases as the adapting concentration increases.

The quickness of adaptation would tend to nullify the effectiveness of contact repellents; however, the corresponding speed of recovery counteracts this. Consequently, the existence of sensory adaptation to chemicals does not appear to be a factor which causes any appreciable reduction in the response of insects to repellents. It would thus appear that the process of adaptation plays no great part in the loss of repellency with time.

Certain characteristics of the response illustrate very beautifully some of the concepts of behavior recently reviewed by Lorenz (1950). In addition to exhibiting the type of adaptation described herein, it parallels in its action many other aspects of behavior seen in vertebrate animals. For example, under continuous stimulation of long duration the fly responds with bursts of activity; it shows an inertia of reaction in that removal of the stimulus, even when followed by rigorous cleansing in water, does not result in immediate cessation of response. On the other hand, proboscis extension *in vacuo*, that is, in the absence of stimulation after prolonged inactivity, has not been observed. The response is fully deserving of more intensive investigation because it offers unusual opportunities for establishing a neural basis for many commonly observed patterns of behavior.

Summary

1. When receptors located in the tarsi of the blowfly *Phormia regina* are stimulated by solutions of sucrose, the insect responds by extending its proboscis.

2. The lowest concentration which would elicit a response from 50% of the population studied was taken as the acceptance threshold. The threshold value obtained depended upon the order in which different concentrations were offered for testing.

3. Adaptation was demonstrated by comparing threshold values obtained (1) by offering solutions in ascending series of concentrations, (2) in descending series, (3) by a random technique, (4) following exposure of the tarsi to subliminal concentrations in one case and supraliminal in the other. The process of adaptation which was measured represented predominantly a state which had been brought about at some level in the central nervous system.

4. The time required for adaptation increased as the logarithm of concentration. For sucrose it varied from one to 13 seconds. Stimulation by mixtures of glycol (unacceptable) and fructose (acceptable) resulted in adaptation to each component.

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A STUDY OF RADIOPHOSPHATE UPTAKE IN PARAMECIUM MULTIMICRONUCLEATUM

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It was our original intent to find out if it is possible, by the use of radioactive elements, to tag microorganisms such as Paramecium, in order that an adequate evaluation of their place in food chains could more accurately be determined. It is possible under certain conditions to render paramecia sufficiently radioactive with inorganic radiophosphate that single animals will give a definite positive count over background. Thus it should be quite possible by means of simple predation experiments to calculate the utilization of microorganisms in specified situations. Coffin *et al.* (1949) and Hayes and Coffin (1951), by adding P^{32} to small lakes, have established by this relatively simple means that phosphate distribution among members of the community was much more rapid than previously supposed and that there was an extensive exchange of elements between organisms. Phosphate turnover was greatest among the algae. Another interesting fact revealed by their results was that certain plants concentrate P^{32} up to 40,000 times the concentration in surrounding water.

It is important to know to what extent aquatic organisms concentrate ions in their bodies from their environment because of the implications in sewage and radioactive waste disposal problems.

METHODS

The organisms used in these experiments were derived from the cultures used by Professor W. H. Johnson of Wabash College in his studies on the growth of *Paramecium multimicronucleatum* Powers and Mitchell in bacteria-free culture. All cultures were grown as described by Evans (1944). Stock as well as experimental cultures were fed on yeast and grown at room temperature (24–27° C.). At the end of 10 days, the population of paramecia reached a peak of about 450 individuals per ml. During the latter part of the logarithmic growth phase, the P^{32} was introduced as NaH_2PO_4 in weak acid solution. No carrier was added and no buffer was needed since the radioactive solution was diluted from one part in 250 of Osterhout's culture medium to one part in 2500 of Osterhout's. The Osterhout's was buffered with $M/20$ phosphate and adjusted to a pH of 7.0 with $M/10$ NaOH. The pH was measured electrochemically with a Beckman pH meter.

Several cultures for the experiments were grown; the amount of P^{32} added varied from 0.1 to 10.0 microcuries per ml. Organisms were withdrawn at intervals and washed as free as possible of the external radioactive phosphate either by dilution following centrifugations or by pipetting. Supernatant fluid from the last wash was always checked for radioactivity. In some cases, P^{32} was added to unfed cultures, but in all other respects the organisms were treated as described above.

The washed organisms in counted numbers were placed in metal planchets with a minimum of fluid and desiccated; single animals were transferred with $\frac{1}{500}$ ml. of fluid, 10 animals with $\frac{1}{50}$ ml., and 100 animals with $\frac{1}{20}$ ml.

In order to determine if the washing was adequate, one-ml. samples of supernatant fluid from all of the successive washings were tested. Similar tests were carried out on solutions through which paramecia were transferred during washing with pipettes. It was found that no practical reduction in residual radioactivity could be effected after the fifth centrifugation. Even after 12 centrifugations, there remained some radioactivity in the supernatant fluid. Five washings brought the count down to about 10 counts per minute per ml. Kamen and Spiegelman (1948) noted that rapid centrifugation of Rhodospirillum caused considerable leakage of phosphate ions from the cells. This was also true but less so for Chlorella. They state that yeast gives up negligible amounts of P^{32}. Likewise, Moraczewski and Kelsey (1948) found that *Trypanosoma equiperdum* gave up phosphorus continuously especially as the activity of the animals decreased. They associated this with increased permeability as death of the cells approached. However, Labaw *et al.* (1950) state that after inorganic phosphate has been incorporated into the nucleic acid molecule in *Escherichia coli*, there is no interchange of the phosphorus either by metabolic exchange or death.

A certain amount of turnover is normal, but centrifugation seemed to enhance the loss of P^{32} from the cell. It was calculated that the amount of radioactivity in the medium carried over with the paramecia from the last wash to the planchets for desiccation was negligible.

Washing away the radioactive external medium by transferring with micropipettes was more satisfactory when only a few animals were desired, but this method was too slow for washing large numbers of organisms.

In a typical experiment, hundreds of paramecia were drawn out of a radioactive culture in one ml. of medium and deposited in a 15 ml. centrifuge tube. To this was added 13 ml. of fresh balanced salt solution (Osterhout's). The paramecia were centrifuged at 34 gravities for 10–12 seconds. This was sufficient to bring all normal paramecia to the bottom, but starved paramecia required about 300 gravities for 30 seconds. A glass plunger was inserted, one ml. of the medium removed for testing, and the supernatant poured off. Fresh balanced salt solution was again added and the process repeated as many times as was necessary.

Radioactivity was determined by means of a standard 64-scale Geiger counter with a Tracerlab mica window tube 1.9 mg./cm.^{2} in window thickness. The specimens were centered in planchets and counted at a distance of 5 cm. from the center of the tube. Each sample was counted for five minutes. Those with especially high levels of radioactivity were counted for one minute. All counts were corrected for decay.

Results

The presence of the radioactive phosphorus in the paramecia cultures did not appear to affect the course of the cultural history. The organisms remained the same size, their behavior was unmodified and the reproductive rate, though not measured exactly, appeared to parallel control cultures.

In the initial experiment, 0.5 microcurie of P^{32} per ml. was added to a culture of paramecia. The organisms were dividing at a maximum rate. The culture was one week old; there were approximately 350 paramecia per ml. Twenty-four hours after introduction of the P^{32}, paramecia were removed and prepared for radioactive determinations either singly or in groups of ten. While the single paramecia always registered a positive count over background it was low, averaging 3.8 counts per minute. The groups of 10 paramecia per dish showed an increase by a factor of 10 over the singles; this confirms the reliability of the individual counts.

Six days later, the radioactivity of the paramecia was again measured. The activity had increased slightly, being now 4.5 counts per minute per animal. Again the dishes containing 10 animals each showed a very close correlation with the 10 separates.

It is to be noted that the paramecia were feeding and possibly most of the intake of phosphate was through the food vacuole system, either in solution or in the food organisms.

Several new cultures, each containing thousands of thoroughly washed paramecia, were established. These received no food. Radiophosphorus was added and radioactive determinations were made at intervals (after 20 hours, 3 days, 5 days, 9 days and 11 days). The concentrations tested were 0.1, 0.2, 0.5, 0.8 and 1.0 microcuries per ml. Three cultures were set up at each of these concentrations.

The results of these experiments showed that paramecia which have no available food take in very little P^{32}. In fact, groups of 100 paramecia averaged only 8.3 counts per minute for an average of 0.08 counts per animal per minute. There was no difference in uptake between the weakest and greatest concentrations of radioactive substance. Time had little effect; the organisms were about as radioactive after 20 hours as at the conclusion of the experiment.

On the eleventh day, yeast was added to two of the cultures containing 1.0 microcurie of P^{32} per ml. The population soon began to increase. Twenty hours later, radioactivity counts nearly equaled those of paramecia in the initial experiment.

Inasmuch as many microorganisms, especially green forms, may concentrate inorganic ions inside the cell, it might be revealing to render old cultures of paramecia radioactive. Andresen *et al.* (1950), using C^{14}, were able to concentrate this element in *Stentor polymorphus* containing symbiotic Chlorella. The Stentor individuals finally yielded 283 counts per minute. Old cultures of paramecia become rust-colored and sometimes green due to the presence of bacteria and Chlorella which the paramecia ingest.

The old cultures chosen were three months old and the paramecia were very numerous and healthy. Four old cultures were selected; to two were added 0.1 microcurie of P^{32} per ml., and to the other two cultures were added 2.0 microcuries per ml. Four new cultures were started and run parallel with the old cultures.

The paramecia of the new and old cultures containing 0.1 microcurie of P^{32} per ml. showed a significant difference in radioactivity. Paramecia from old cultures showed consistently twice the radioactivity as those from new cultures. There was an even greater difference between paramecia taken from new and old cultures containing 2.0 microcuries of P^{32} per ml. Paramecia from the old cultures were five times as radioactive as were those from the new cultures. Apparently much of their phosphate was acquired as food. The results also show that the greater the concentration of radioactive phosphate, the greater the amount of radioactivity in the paramecia. It should be pointed out that paramecia of old cultures are primarily non-dividing.

After absorption, is the phosphate tightly held in the cell or is it readily released? This question was partially answered by a series of tests. A few hundred radioactive paramecia were washed through 10 centrifugations. These were left in one ml. of the last wash. Five such tests were run. One tenth ml. of solution was removed at intervals, and the radioactivity tested. Table I shows the results.

TABLE I

Loss of P^{32} from Paramecium determined by radioactive measurement of 0.1 ml. samples of medium

Time in hours after washing paramecia in fresh medium	Counts per minute of 0.1 ml. of medium
0	1.5
24	64
66	95
116	154
168	167
192	170

The paramecia rapidly release the phosphate. Powers (1947), rendering *Paramecium aurelia* radioactive by feeding them radioactive *Aerobacter aerogenes,* noted a loss of more than one-half of the total phosphorus after 20 hours from the time the cells were separated from the source of radiophosphorus.

DISCUSSION

Paramecium multimicronucleatum can be made sufficiently radioactive with P^{32} to serve in predation experiments. However, in view of the fact that paramecia give up large amounts of radioactive phosphate within a few days, the duration of such experiments must be limited. Perhaps other radioactive substances will be found to remain longer or even indefinitely in the body of Paramecium. One interspecies experimental population, using radioactive paramecia as prey and *Didinium nasutum* as predator, has shown that the radioactivity of the prey is taken over almost if not entirely by the predator, and that the radioactivity of a dividing Didinium seems to be about equally distributed between the two daughter cells.

In natural populations, the amount of food present for Paramecium would be a deciding factor in uptake of P^{32}. Unfed paramecia seem unable to concentrate inorganic phosphate. Phosphorus is required for metabolism by microorganisms (Elliott and Hunter, 1951; Sullivan, 1950; Weisz, 1949), and the experiments reported herein indicate that most of the phosphate obtained by *Paramecium multimicronucleatum* is acquired in its food. Probably small amounts are absorbed directly and some adsorbed on the surface. Saprophytic and autotrophic forms seem to be able to absorb large amounts of phosphate directly.

Using Popoff's (1909) equation for determining volume of *P. caudatum,* it was calculated that paramecia which are feeding have roughly 20 times more radioactivity than the surrounding medium, while unfed paramecia have less than one-half the radioactivity. Mazia and Hirshfield (1950) measured the uptake of inorganic P^{32} by Amoeba in the absence of food, and they found that the concentration of phosphate in the cell was greater than outside by a factor of at least 50. It is probable that, as in *P. aurelia* (Powers, 1947), the phosphate is present in *P. multimicronucleatum* as organic phosphate.

SUMMARY

1. In a medium containing inorganic radioactive phosphorus, *Paramecium multimicronucleatum* become sufficiently radioactive for use in quantitative predation experiments.
2. It is possible to measure the P^{32} uptake of a single individual.
3. When food is absent, Paramecium does not take in inorganic phosphate in solution. The phosphate is acquired in measurable amounts in its food.
4. The phosphate taken in is rapidly lost from the animals, probably as a result of an organic turnover.
5. Paramecia in old cultures, especially cultures containing Chlorella, become more radioactive than those in other cultures probably because the Chlorella which are ingested by the paramecia, absorb much phosphate, and also because old cultures of paramecia are primarily non-dividing.
6. The greater the initial concentration of P^{32} in solution, the more radioactive the paramecia become.
7. When washed by centrifugation, there is a leakage of P^{32} from the organisms over and above the normal turnover of phosphate.

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FURTHER NOTES ON THE TURBELLARIAN FAUNA OF THE ATLANTIC COAST OF THE UNITED STATES

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Since my previous publications on the larger Turbellaria of the Atlantic coast of the United States (Hyman, 1939a, 1940, 1944), very little in the way of new material has come to hand. Undoubtedly much remains to be done on the smaller Turbellaria of the region in question—acoels, rhabdocoels, and alloeocoels—but one may assume that our knowledge of the larger forms, especially polyclads, is practically complete. Florida remains the only region from which additional species may be expected. The present paper aims to put on record some new material and information and to call attention to necessary nomenclatorial changes. As the classificatory position and familial and generic definitions for the following forms were given in the publications cited it appears unnecessary to repeat them here.

Order Polycladida

Family Plehniidae

Plehnia ellipsoides (Girard), 1854, new combination

Discocelides ellipsoides Hyman, 1940

In connection with the finding of a new species of *Plehnia* on the Californian coast I am giving some discussion of the family Plehniidae and its genera that leads me to the desirability of transferring *Leptoplana ellipsoides* Girard, which I determined some years ago as a member of the Plehniidae, to the genus *Plehnia*. I now feel that it fits better in this genus than in *Discocelides* to which I previously assigned it.

Family Cryptocelidae

Phaenocelis purpurea (Schmarda), 1859

Comprostatum insularis Hyman, 1944

At the time of my description of this cryptocelid from the Florida keys, I considered it a new species representative of a new genus for which I created the name *Comprostatum*. Dr. Stephen Prudhoe of the British Museum (Natural History) kindly called my attention to the genus *Phaenocelis* Stummer-Traunfels, 1933. Stummer-Traunfels in 1933 re-investigated the types of Schmarda (1859) and determined that Schmarda's species *Leptoplana purpurea* from Jamaica is in fact a cryptocelid to which he gave the generic name *Phaenocelis*. There can be no doubt that my genus *Comprostatum* is identical with *Phaenocelis* and after some hesitation I am also now of the opinion that my species is identical with that of Schmarda. Hence the name *Comprostatum insularis* becomes a synonym of *Phaenocelis purpurea*.

Family Leptoplanidae

Zygantroplana angusta (Verrill), 1892, new combination

Stylochoplana angusta Hyman, 1939

In re-investigating from the original material *Leptoplana angusta* Verrill, 1892, now known to be native to the southern part of the Atlantic coast, I tentatively placed it in the genus *Stylochoplana* while recognizing some lack of conformity with this genus. Dr. Ernesto Marcus of the University of São Paulo, Brazil, kindly called my attention to the genus *Zygantroplana* Laidlaw, 1906, which I had unfortunately overlooked. Verrill's species fits perfectly into this genus and hence its name becomes *Zygantroplana angusta.* It is interesting to note that a species of *Zygantroplana,* so similar to *angusta* that it should probably be regarded as a geographic variant of the latter, is very common on the Brazilian coast near São Paulo (Correa, 1949). This would seem to be the first instance of a similarity in the Turbellarian fauna between Florida and Brazil.

Family Pseudoceridae

The genus Thysanozoon in Florida

The genus *Thysanozoon* is probably the easiest of all polyclad genera to recognize since the clothing of the dorsal surface with numerous, closely placed, conspicuous papillae is, by definition, diagnostic of the genus. By the same token, the species of the genus are particularly difficult to differentiate because of a general similarity of internal structure. Color becomes of considerable importance in differentiating the species and this is lost in preserved specimens. In 1851 Girard described under the name *Thysanozoon nigrum* a single specimen of a black *Thysanozoon* that he had taken on the eastern coast of Florida. Verrill (1901) recorded what appears to be Girard's species from Bermuda and during a stay at Bermuda in 1935, I also took two specimens alive (Hyman, 1939b). The Bermuda specimens were a rich velvety black throughout the dorsal surface with some small white flecks. Sections of the copulatory apparatus showed distinctive characters. *T. nigrum* was not again taken on the Florida coast, however, until 1950 when Dr. Harold Humm, in charge of the Florida State University marine station at Alligator Harbor near Tallahassee, kindly sent me a living *Thysanozoon* from that locality, collected February 4. This was 40 mm. long, of an oblong shape when crawling extended, and of a general dark grayish black, with white flecks on the margins and papillae. The animal was fortunately sexually mature and sections showed it to be identical with the Bermuda specimens. *Thysanozoon nigrum* therefore occurs both on the Florida coast and at Bermuda. It is a valid species distinguishable by the uniform black color of both dorsal surface and papillae, by the paired male apparatuses, by certain details of the male apparatus described in my Bermuda article, and by the presence of an intestinal branch in each papilla. Stummer-Traunfels (1895) in a review of the genus *Thysanozoon* pointed out that part of the species have but one male apparatus and that the occurrence of an intestinal branch in each papilla was known only for the type species, *T. brochii.* Marcus (1949) in reviewing the valid species of the genus records 9 with single and 15 with double male apparatus but does not mention the question of the presence of an intestinal branch in the papillae except with regard to his new species in which such branches are wanting.

There is another Floridan species of *Thysanozoon* besides *nigrum*. Pearse (1938) records taking no less than 29 specimens of a *Thysanozoon* in Crooked Island Sound and St. Joseph's Bay, localities in the same general part of Florida as Alligator Harbor. Pearse identified these specimens as *T. brochii* but described only the color which varied from cream to brown and purple with brown and purplish papillae. A whole mount of one of these specimens has come into my possession but as it appears immature (length 20 mm.) has not been sectioned. The extension of an intestinal branch into each papilla is clearly seen upon the whole mount, which thus does resemble *brochii* in this respect. What seems to be a specimen of the same species has been received for identification from the United States National Museum. This was collected by F. M. Bayer, January 26, 1951, under stones along the County Causeway, Biscayne Bay, Florida, which lies along the southern end of the eastern coast. This specimen also unfortunately proved sexually immature but in the cleared worm the intestinal network sending a branch into each papilla was evident. The specimen was accompanied by a color description which stated that the animal was purplish maroon with brown papillae and some white spots along the edge and on the papillae. There would seem little doubt that this specimen is identical with those of Pearse but whether they can be regarded as *T. brochii* remains uncertain.

Still another preserved specimen was received from the Florida State University, collected in November, 1938, at Clearwater, Florida, located at about the middle of the western coast of the peninsula. This specimen had been bleached white by the preservative and was also immature so that it proved of no value. Whether the intestinal branches extend into the papillae could not be determined.[1]

Family Euryleptidae

Prostheceraeus maculosus (Verrill), 1892, new combination

Eurylepta maculosa Verrill, 1892

In previous comments on this species I remarked that some good specimens were necessary before its systematic position could be determined with certainty. Apparently the species had not been refound since Verrill's publication. Therefore I was very pleased to be informed by Dr. Chauncey Goodchild of the staff of Invertebrate Zoology at Woods Hole that he had found the species in some abundance on wharf pilings at Lagoon Pond Bridge, Martha's Vineyard. On August 2, 1949, Dr. Goodchild kindly brought me a number of live specimens from that locality and also very generously took the trouble to fix a number of them for me. The living animal (Fig. 1) is about 10 mm. long when moving extended, and of an oblong or elongated oval shape with two pointed tentacles at the anterior margin. It sails along rapidly over the seaweeds by undulating the body margins after the usual

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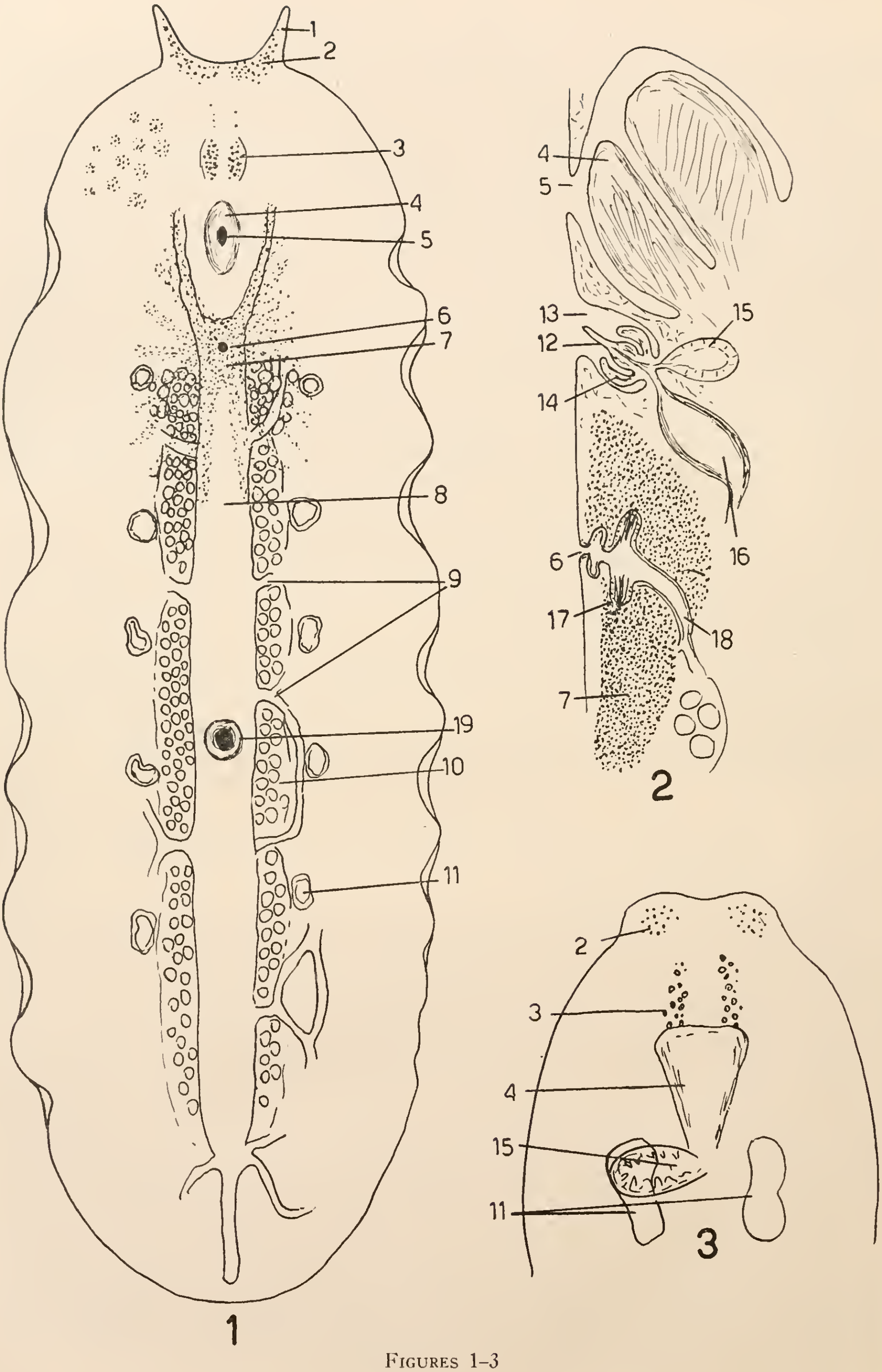
[1] Since the above was sent to press there has been received from the U. S. National Museum a vial of polyclads collected in the Biscayne Bay region of Florida by F. M. Bayer. This vial contains another specimen of *Thysanozoon nigrum*, and two specimens of *Pseudoceros crozieri* Hyman, 1939, hitherto known only from Bermuda and thus furnishing a second case of a polyclad common to Bermuda and Florida; further two specimens of the *Acerotisa* mentioned above, clearly an undescribed species, and an undescribed leptoplanid.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Figures 1–3

manner of cotylean polyclads. The color is light tan dotted with dark brown spots. Numerous eyes are present along each tentacle and on the body margin between the tentacle bases; there is also a pair of cerebral eye clusters over the brain region. Examination of pressed live specimens showed at once that the intestinal branches are anastomosed into a network and this puts the species in the genus *Prostheceraeus.* Several specimens were sectioned and these also show the characters of *Prostheceraeus.* Some details from these sections have been added to Figure 1. The main intestine forks anteriorly around the pharynx and behind this gives off irregularly several branches on each side that anastomose into a network. Along either side of the main intestine is seen an elongated tubular uterus stuffed with eggs and this gives off laterally uterine vesicles. In a specimen sectioned frontally five of these vesicles were seen on each side alternating with the main intestinal branches as characteristic of the genus. The sucker is situated at about the body middle. A sagittal view of the male and female copulatory apparatus is shown in Figure 2 but offers nothing particularly distinctive. It is unfortunate for systematic purposes that the copulatory apparatuses are very similar throughout the Cotylea, a fact that adds to the difficulties of specific identification in this group. A whole mount of the species has been deposited in the American Museum of Natural History.

The genus *Acerotisa* on the Atlantic coast

In 1940 I described *Acerotisa baiae* from a single specimen collected by Pearse in St. Joseph's Bay, Florida. I have here to record the unexpected finding of a specimen of this species by Dr. Chauncey Goodchild at Lagoon Pond Bridge, Martha's Vineyard, August 13, 1948. Alive the specimen was egg-shaped with pointed anterior end and of a translucent white color with the dorsal surface dotted with cream-colored dots. One may also expect to find from Woods Hole southward in the floating *Sargassum* the minute species *Acerotisa notulata* (Bosc) 1801, of which I gave a description in my article on Sargassum Turbellaria (Hyman, 1939b). Still another *Acerotisa* species occurs on the Atlantic coast of the United States. I am again indebted to Dr. Harold Humm for sending a living specimen of an *Acerotisa* obviously different from the foregoing species. This was collected April 16, 1951, at Alligator Harbor, Florida. Unfortunately it arrived partly disintegrated; what remained is shown in Figure 3. The numerous eyes in both the marginal and cerebral clusters clearly differentiate the animal from any other *Acerotisa* species known from the Atlantic coast or Gulf of Mexico. The animal was white peppered with dark dots. The animal bears a striking resemblance to *Acerotisa piscatoria* Marcus 1947, collected near São Paulo, Brazil, but the identity cannot be established without further material.

FIGURE 1. *Prostheceraeus maculosus*, from life, some details added from sections.

FIGURE 2. Median sagittal section of pharyngeal region of *Prostheceraeus maculosus*, showing male and female copulatory apparatus.

FIGURE 3. Anterior part of an undescribed species of *Acerotisa* from the Gulf coast of Florida, from life.

1, tentacles; 2, tentacular eyes; 3, cerebral eyes; 4, pharynx; 5, mouth; 6, female gonopore; 7, cement glands around female canal; 8, main intestine; 9, intestinal branches; 10, uterus; 11, uterine vesicles; 12, penis papilla; 13, male gonopore; 14, penis sheath; 15, prostatic vesicle; 16, seminal vesicle; 17, cement pouch of female canal; 18, vagina; 19, sucker.

Order Tricladida

Note on *Probursa veneris*

In 1944, I described the interesting marine triclad, *Probursa veneris*, found sheltering in empty *Venus* shells on a sand bar near the western end of the Atlantic shore of Long Island. It was therefore very surprising to receive a number of live specimens of this worm from Dr. Harold Humm, who had found them in Bogue Sound, near Beaufort, North Carolina, June 20, 1946, on the fronds of a red alga, *Mitophyllum medium*. Not only the adults but also many young and spherical orange capsules were present on the alga. Alive the worms were translucent white, very active and restless, swimming by a twittering motion, and crawling in leechlike fashion by alternately attaching the body ends, that must be provided with adhesive glands.

I wish here to thank Dr. Harold Humm and Dr. Chauncey Goodchild for the constant interest they have shown in my specialty and for their generous expenditure of time and trouble in furnishing me with turbellarian specimens.

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STUDIES ON ARTHROPOD CUTICLE. VIII. THE ANTENNAL CUTICLE OF HONEYBEES, WITH PARTICULAR REFERENCE TO THE SENSE PLATES[1,2]

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The present study was initiated to investigate the question of whether or not the cuticle over chemoreceptors differs other than in thickness from adjacent areas of the cuticle. Due to the mixed distribution of various types of sensilla there is no case where identification of a particular type of sensillum as a chemoreceptor has been absolutely proven. Of the several cases where strong experimental evidence supports the assigned function of chemoreception (Dethier and Chadwick, 1948), the sense plates of honeybees were chosen for study largely because preliminary investigation showed they were more readily prepared for electron microscope examination than other sensilla. Examinations made about ten years ago showed that probably little useful information would be obtained by electron microscope examination of untreated cuticles overlying chemoreceptors; what was needed was an accessible type that could be readily prepared in order to develop extraction and staining analyses for the electron microscope to parallel studies made with the light microscope. Since a single tangential section of a bee's antenna may show several dozen surface views of sense plates satisfactory for electron micrography, these are favorable if not indeed ideal material for such a study.

The answer obtained to the initial question stated above is that the cuticle over these sense plates is different from the cuticle over other parts of the antenna in more ways than just in thickness. Also, the cuticle differs on different types of sensilla. Little information is available on the nature and significance of these qualitative differences but the relatively low lipid content of the epicuticle and the incomplete sclerotization imply relatively ready penetration. Incidentally, an unexpected and novel sequence of sclerotization changes was encountered.

METHODS

For this study, honeybees were mostly collected from hives maintained here. The majority of the work was with the worker caste but, at least for electron micro-

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[1] Paper No. 2831, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul 1, Minnesota.

[2] The work reported in the present paper was done under a contract between the Office of the Surgeon General, U. S. Army, and the University of Minnesota. Under the terms of this contract the Army neither approves nor restricts the publication of data.

The author would like to express his indebtedness to Frances H. Korda, Helen T. Y. Lee, C. L. Pu and Marion B. Clausen for considerable amounts of technical assistance at various stages of the work. Chemists of the du Pont Company suggested that $TiCl_4$ and $ZrCl_4$ would react with exposed amine groups on chitosan, and supplied us with samples. Drs. M. H. Haydak and T. A. Gochnauer kindly kept us supplied with bees of desired ages. Thanks are also due to Drs. V. G. Dethier, M. H. Haydak and D. E. Minnich for reading the manuscript.

graphs of sense plates, the fully hardened adult drone is similar. Pupal development requires about nine days, and the adult bee emerges from the pupa within a sealed cell and does not chew its way out of this cell until about a day after emergence. For purposes of the present paper, a "teneral adult" is defined as an adult which has been removed from its pupal cell prior to chewing its way out, and an "older adult" is defined as an adult active outside the hive.

Pupae were seriated in terms of developmental stage without any record being kept of the actual hours of development. Ample material being available, sealed pupal cells were opened at random and preliminary seriation made on basis of the eye color, younger stages being more finely seriated by examining the head capsule under a dissecting microscope. Excessive numbers were taken in the desired ranges and finally seriated after sectioning.

Examination of preparations was made with a Leitz microscope equipped with apochromatic lenses, a Leitz research polarized light microscope, or an RCA electron microscope, model EMU. Some preparations were also examined by phase-contrast microscopy. A considerable variety of techniques was employed. Sections were made free hand from fresh material or with a microtome after embedding either *in vacuo* following freeze-drying or *via* benzene following fixation (usually in Carnoy's or Bensley's fluid but sometimes in Bouin's fluid). For the ordinary light microscope, sections were examined unstained, or after extraction with hot water (100° C.) or 10% NaOH solution, or after staining with Mallory's triple stain, Heidenhain's iron haematoxylin, the argentaffin reaction, the Schiff polysaccharide test, or 2% osmic acid. For the polarized light studies, sections and whole mounts were examined in water both as normals and after treatment with 10% NaOH at 100° C. to purify the chitin, or 60% KOH at 160° C. to convert the chitin to chitosan.

For the electron microscope, free-hand tangential sections were prepared, the cells lysed by brief soaking in distilled water, the inner surface then brushed to wipe off the disintegrating cells, the sections rinsed in distilled water, and then, with or without further treatment, placed on bare electron microscope grids and air-dried. This method of preparation limits the EM examination to a single view—that normal to the plane of the surface. Treatments included extraction procedures similar to those used previously in cuticle studies (Richards and Korda, 1948) and an extensive series of reagents treated in the next section. It was noted that in general, less disruption of extremely thin membranes and ultrafine structures followed purification to chitin when Diaphanol[3] was used than when hot alkaline solutions were employed.

Electron Microscope Staining of Cuticle

Objects are resolved in an electron microscope because of differences in density between the object and its surrounding medium. A common limitation in biological work is that inadequate density differences between adjacent parts result in inadequate contrast in the pictures obtained. In general, this limitation may be overcome by increasing the purification of the material, by shadow-casting, or by inducing the deposition of heavy elements in the material. Purification is of limited value when one is studying the normal structure of a heterogeneous system, and shadow-casting is limited to clarifying surface irregularities (best with one type

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[3] A saturated solution of chlorine dioxide in 50% acetic acid.

TABLE I

Electron microscope staining reactions with various cuticle samples

Aqueous sols.	% conc.	Chitin *Periplaneta* trachea	Peritrophic membrane *Phormia*	Larval cuticle *Aedes*	Trachea *Periplaneta* adult	Trachea *Phormia* adult	Trachea *Calandra* adult	Crop lining *Phormia*	Crop lining *Periplaneta*	Wing membrane *Aedes*	Wing scales *Aedes*	Tracheal airsac honeybee	Sense plates honeybee
$FeCl_3$	2	—	sl. Gr.	—	?	?	—	?	?	Gr.	—	sl. Gr.	—
$ZrO(NO_3)_2$	0.1–5	—	v. Gr.	v. Gr.	Gr.	sl. D.	—	sl. Gr.	sl. D.	sl. Gr.	—	sl. Gr.	sl. Destr.
$AgNO_3+NH_4OH$	*ca.* 5	—	?	—	var. Sp.	v. l. Sp.	—	—	?	v. sp.	v. l. Sp.	—	f. Sp.
$H_3PW_{12}O_{40}$	2+6	Destr.	?	?	v. Retic.	sl. D.	?	Gr.	?	—	—	var. Gr.	Gr.
OsO_4	2	—	—	sl. D.	v. D.	sl. D.	?	?	sl. D.	v. D.	v. D.	v. D.	sl. D.
$IrCl_4$	1	—	v. Gr.	v. Gr.	sl. Destr.	sl. Gr.	sl. Gr.	sl. D.	sl. Destr.	?	?	v. Gr.	—?
K_2PtCl_6	2	—	sl. Destr.	—	—	—	—	?	?	—	—	sl. Gr.	—
$HgCl_2$	Sat.	—	?	Var.?	—	—	—	—	?	—	—	sl. Gr.	—
Millon reagent	—	—	—	—	—	—	—	—	sl. Destr.	—	—	—	?
$Pb(C_2H_3O_2)_2$	2	—	sl. D.	—	D.	—	?	—	?	—	—	—	—
$Th(NO_3)_4$	2	—	sl. D.	Gr.	v. Retic.	sl. D.	sl. D.	sl. D.	sl. D.	sl. Gr.	—	Gr.	?
$UO_2(NO_3)_2$	2	—	?	?	—	—	—	sl. D.	?	—	—	var. Gr.	—
$NaZn(UO_2)_3$ $(C_2H_3O_2)_9$	2	—	sl. D.	sl. Gr.	v. Retic.	?	sl. Gr.	sl. Gr.	?	sl. Gr.	?	Gr.	—

— = negative; ? = negative or questionable slight effect; D. = distinct darkening, more or less general; Gr. = granular appearance to membrane; Sp. = distinct separated spots; Retic. = spots connected by streaks giving reticulation; Destr. = some structural destructive changes.

v. = very; sl. = slight; f. = fine or minute; l. = large; var. = variable or inconsistent.

of surface discontinuity on an otherwise plane surface; shadow-cast specimens were examined but did not facilitate study of these sense plates). Accordingly, a considerable amount of effort was expended in an attempt to develop a useful set of "stains" which would augment contrast *within* a specimen of cuticle and perhaps even be sufficiently specific in action to aid in cytochemical identifications (Richards, 1952a). The results were discouraging but not nil.

Some of the more interesting data are presented in Table I. These results were obtained after cleaning cuticles in distilled water and then soaking in the reagents at the indicated concentration (usually two days), excess of reagent being removed by washing in water prior to drying on an electron microscope grid. All tests were both replicated and repeated. Inconstancy in thickness of preparations, even in only slightly separated parts of the same membrane, makes it impossible to distinguish between truly negative effects and a possible uniform deposit by comparing control and treated membranes. But structural variability is much more serious than variations in thickness; recordable differences are limited to relatively gross qualitative differences (see Richards and Korda, 1948).

Since no two of the membranes selected as test objects gave identical results, the data emphasize that a considerable amount of interspecific variation is to be expected in the details of cuticle chemistry. The antennal sense plates are not identical in reactions to any of the other membranes tested, but they appear in these tests to be more similar to the cuticle samples from *Phormia* than to the tracheal airsac walls or, as will be brought out in later sections, other cuticularized areas of the same species.

The osmic acid effect (Fig. 23) is negative after extraction of the membrane with hot chloroform. At least for the honeybee airsac it is also negative after exposure to intense electron bombardment (see Hillier *et al.*, 1950). Occasional breaks in the specimens show that the effect is localized in the epicuticle but no separable osmium-positive layer was recognized. Broken preparations of bee airsacs show two principal and separable layers; both the osmic acid and the iridium tetrachloride effects are limited to the outer of these layers but the outer layer itself is still present after refluxing with chloroform and will still react with the iridium chloride. In fact, series of tests with cockroach tracheae, bee airsacs, bee sense plates and mosquito wings and scales showed that extraction with hot chloroform interfered only with the osmic acid reaction. Presumably the osmium is reduced by only the lipids in the cuticles used for these EM tests, and the other compounds react with non-lipid components.

The ammoniacal silver nitrate effect is the standard argentaffin reaction positive for polyamines, polyphenols and certain aldehydes. In cuticle work, a positive reaction is presumed to be due to the presence of a polyphenol substrate for sclerotization (Richards, 1951). The degree of localization of the polyphenol by means of this test is not known but the spots of silver (Fig. 20) are clearly discrete in contrast to the diffuse deposition of osmium (Fig. 23).

Except for the osmic acid and ammoniacal silver nitrate reactions, no explanation can be given for the staining of cuticle by compounds listed in Table I and illustrated in Figures 20–23. Obviously, however, the reactions are not simple ion effects, for the nitrate of thorium is excellent but the chloride is not, and of the three uranyl compounds tested, only the complex one gave good pictures. Several routine histological reagents (chloroplatinic acid, mercuric chloride) were either negative or so light and general that the effect was not visible. Aqueous solutions of all of the compounds in Table I are acidic except for the ammoniacal silver nitrate which is close to neutrality. Phosphotungstic acid is a general protein precipitant that deposits in at least most of the cuticles used but the alkaline solutions of sodium and potassium tungstate are ineffective; however, many of the ineffective compounds form acidic aqueous solutions and may even be known protein precipitants.

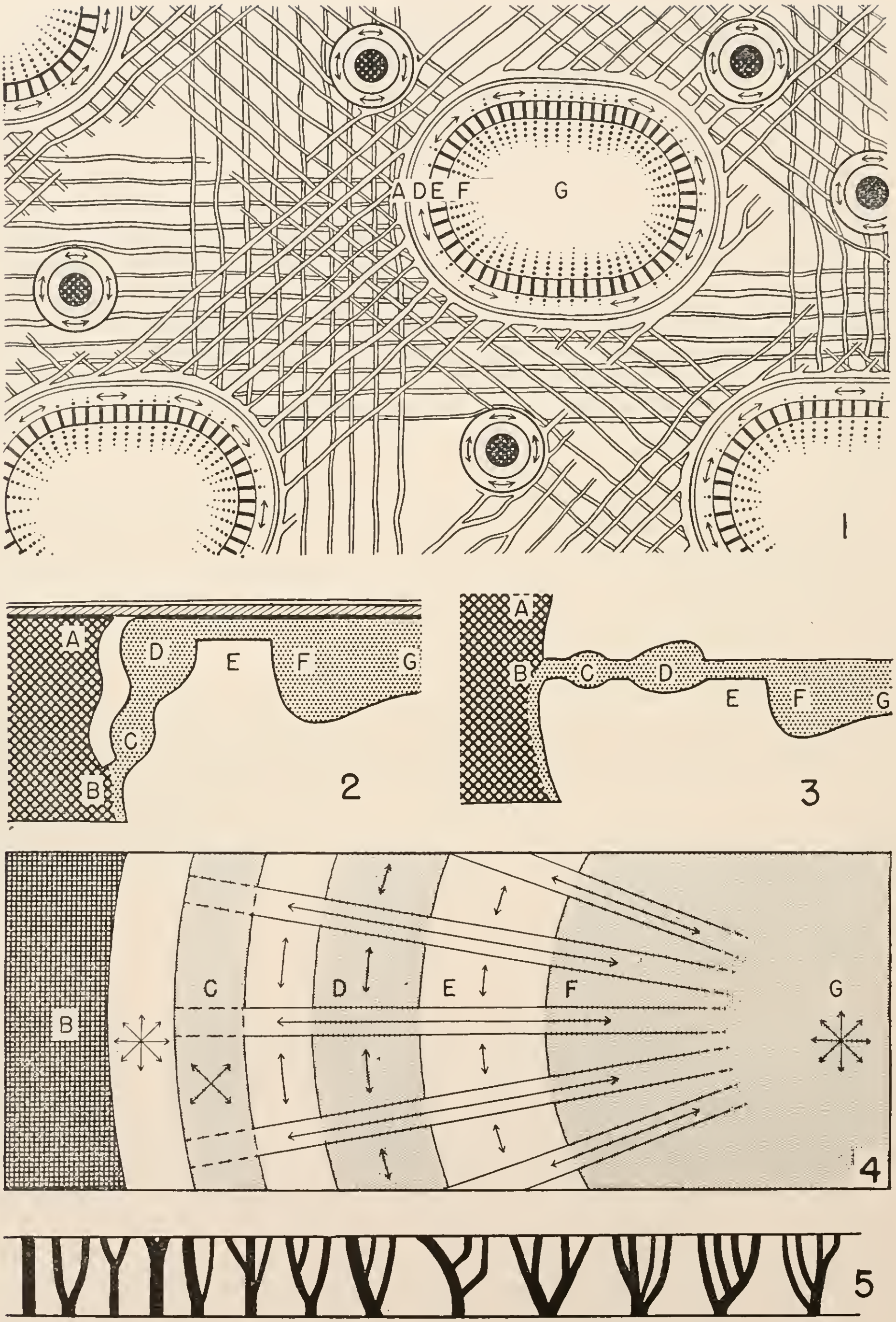
No stain for chitin was found. Since the honeybee airsac is negative to the classic tests for chitin (Richards, 1951), the EM stains do not suggest the possibility of a stain which will react with chitin in cuticle but not with chitin after purification. Chitin and chitosan are both visibly colored by aqueous solutions of iodine plus potassium iodide, but in the electron microscope this treatment results only in a slight overall increase in density which does not seem helpful. It is interesting to note that the chitin chains may not only be de-acetylated to chitosan but also oxidized to the polyaldehyde with periodic acid (involving removal of entire acetylamine side groups) without the micelles appearing different in electron micrographs.

Chitosan can be readily stained by the use of zirconium or titanium tetrachloride in anhydrous organic solvents.[2] In some cases, treatment with these can be used to augment the clarity of micelles (compare Figs. 27 and 29) but the pretreatment required to produce chitosan is violent and leaves little general use for the reagents. Applied to normal cockroach tracheae, these compounds had a somewhat destructive effect or caused dissolution of the endocuticle, depending on the solvent employed; they seemed to give no staining effect to normal cuticle.

Other compounds which have been tested as aqueous solutions (usually at 2%) and found not promising as EM stains for these test cuticles include: NaCl, KCl, $\mathrm{CaCl_2}$, $\mathrm{CrCl_2}$, $\mathrm{MnCl_2}$, $\mathrm{MnSO_4}$, $\mathrm{KMnO_4}$, $\mathrm{K_3Fe(CN)_6}$, $\mathrm{K_4Fe(CN)_6}$, $\mathrm{FeSO_4}$, $\mathrm{CoCl_2}$, $\mathrm{Na_3Co(NO_2)_6}$, $\mathrm{NiCl_2}$, $\mathrm{CuSO_4}$, $\mathrm{(NH_4)_6Mo_7O_{24}}$, $\mathrm{AgNO_3}$, $\mathrm{CdCl_2}$, $\mathrm{SnCl_4}$, $\mathrm{KSbOC_4H_4O_6}$, $\mathrm{K_7TeO_3}$, KI, $\mathrm{H_5IO_6}$, $\mathrm{KIO_4}$, CsCl, $\mathrm{Cs_2SO_4}$, $\mathrm{BaCl_2}$, $\mathrm{Na_2WO_4}$, $\mathrm{K_2WO_4}$, $\mathrm{KAu(CN)_2}$, $\mathrm{HgBr_2}$, HgCl, $\mathrm{TlNO_3}$, TlI, $\mathrm{Pb(NO_3)_2}$, $\mathrm{NaBiO_3}$, $\mathrm{BiC_6H_5O_7}$, $\mathrm{ThCl_4}$, and $\mathrm{UO_2(C_2H_3O_2)_2}$. $\mathrm{SbCl_3}$ was tested as a saturated solution in chloroform. Several of these compounds are to a greater or less extent destructive to cuticular membranes ($\mathrm{KMnO_4}$, $\mathrm{SbCl_3}$, $\mathrm{KAu(CN)_6}$, etc.).

GENERAL DESCRIPTION OF THE ANTENNAE

The antennae and antennal sense organs of honeybees have been described by a number of authors (see Snodgrass, 1925). The antennae of the worker caste consist of twelve segments with freely movable membranous joints between each segment. The sense plates or sensilla placodea are found only on the distal eight segments, but on these segments are rather densely packed in a band extending from near the basal rim to near the apical rim and circumferentially approximately two-thirds of the distance around the segment. Bands of sense plates are similarly located on each of segments 5–12. In effect, then, there is a band of sense plates extending the length of these combined segments except for interruption at the joints, and a corresponding band free of sense plates on the outer surface. Setae called "sense hairs" are found both scattered around between sense plates and on the band that is free from sense plates. Other types of sensilla are more local in


A
D
E
F
G
1
A
D
E
F
G
C
B
2
A
B
C
D
E
F
G
3
C
D
E
F
G
B
4
5

Figures 1–5

distribution. It has been estimated that the antenna of the worker caste has 5000–6000, those of drones about 30,000 sense plates, and that the total number of antennal sense cells is of the order of 500,000 (Snodgrass, 1925).

We can use the above facts, plus data to be presented, as basis for recognizing six types (or areal types) of cuticle on the antenna: (1) sclerotized cuticle between sense plates, (2) sclerotized cuticle in the band devoid of sense plates, (3) sclerotized cuticle devoid of sense plates and setae and forming the basal and apical rims of each segment, (4) soft or unsclerotized cuticle on the intersegmental joints, (5) sclerotized cuticle of sense hairs and sense pegs, and (6) less sclerotized cuticle on sense plates and "sunken setae." Any of these may be compared with cuticle from other parts of the body—such comparison showing that the unorthodox sclerotization of the antennae is not found in the general cuticle of thorax and abdomen and hence is peculiar to the antennae as such, rather than to the honeybee as a species.

Actual presentation of the data, however, will be simpler or at least less redundant if we treat (1) the chitin chain or micelle orientations in the general antennal cuticle, (2) orientations in setae, (3) general structure and orientations in sense plates, (4) development of the epicuticle, (5) development of the procuticle, (6) comparison of the cuticle on various types of sensilla, and (7) comparison to thoracic and abdominal cuticle.

MICELLE ORIENTATIONS IN THE GENERAL CUTICLE

Like arthropod cuticle in general (Richards, 1951), the antennal cuticle is laminated. But the laminae in the bee antennae are thin (ca. 0.8 μ) and so low in contrast that they cannot be readily seen in routine observation except at the antennal joints where the cuticle shows a light staining with fuchsin between the thickened laminae (Fig. 31). Unstained sections examined by dark phase-contrast microscopy reveal a general laminate structure. Laminations have been discerned in some sections of frozen-dried preparations stained with haematoxylin. Laminations may also be inferred from alternating lighter and darker bands of birefringence in sections treated with hot water or alkali (Fig. 8), from the presence of crossed Balken layers (see below), and from rows of spots along pore canals of antennae treated with ammoniacal silver nitrate (Fig. 12). The fully developed cuticle is approximately 15 μ thick; accordingly there are some 15–20 laminae.

In the normal, fully developed cuticle no macrofibers or Balken can be seen

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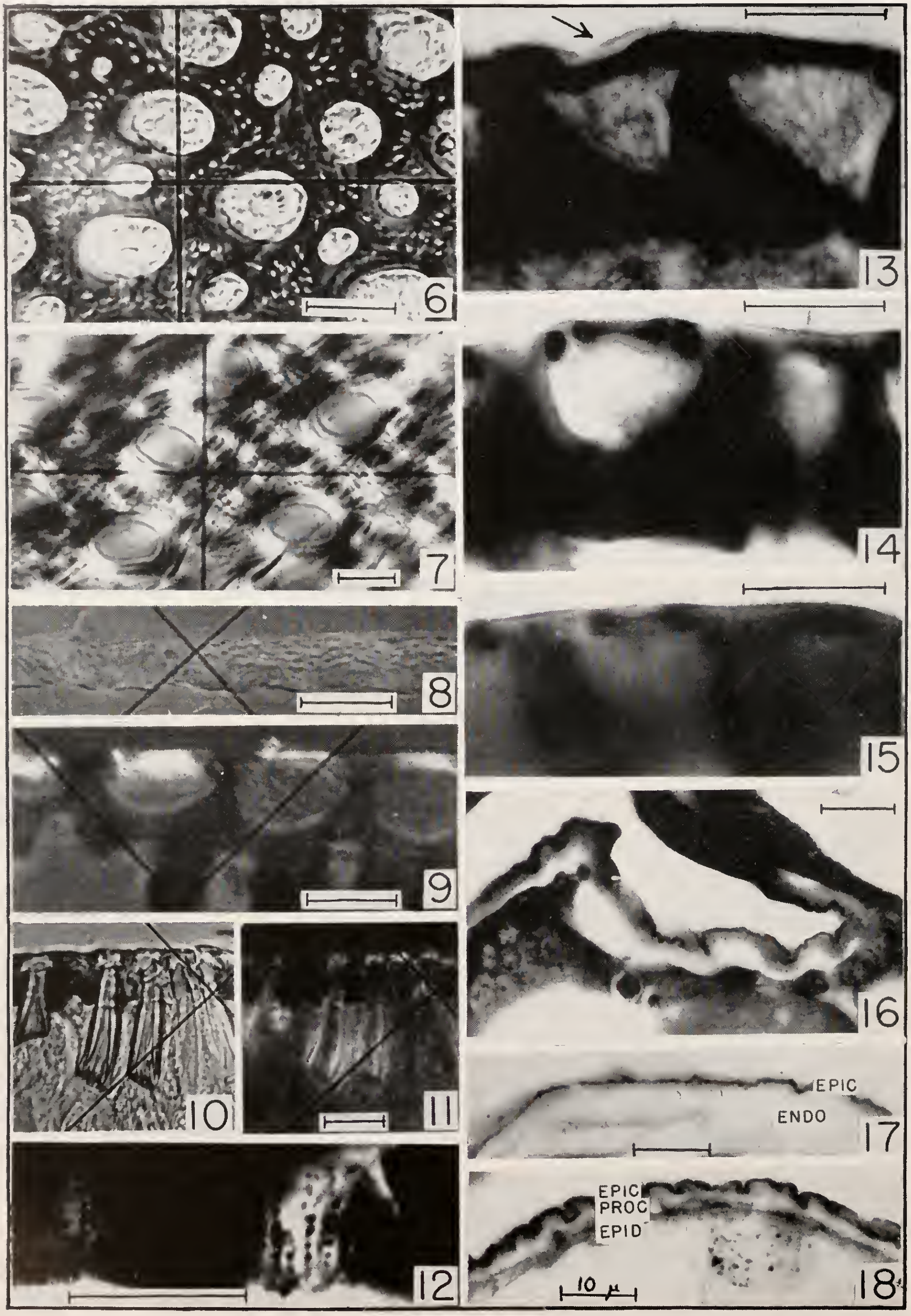
FIGURE 1. Semi-diagrammatic sketch of a small area of an antennal segment showing distribution of sense plates and sense hairs (sockets as cross-hatched circles), orientations of Balken as seen in specimens purified for chitin, and orientations of micelles in sockets as indicated by arrows. Lettering indicates corresponding parts in Figures 1–4.

FIGURE 2. Diagrammatic section of normal relations of the cuticle of half a sense plate, its rim or socket, and the adjacent cuticle.

FIGURE 3. Similar diagrammatic section showing opening out of structure, concomitant with drying, after rupture or removal of the epicuticle. Needed for interpretation of some of the electron microscope pictures (Figs. 19 and 25).

FIGURE 4. Map of micelle or chitin chain orientations in the cuticle of a sense plate and its socket.

FIGURE 5. Types of ribs or Balken seen in electron micrographs of the thin membrane between sense plates and the socket rim (see Figs. 19 and 22).


6
7
8
9
10
11
12
13
14
15
16
EPIC
ENDO
17
EPIC
PROC
EPID
10 μ
18

Figures 6–18

clearly (Fig. 6), and the cuticle seems nearly isotropic (Fig. 9) (except for setae and sockets, see next section). If the cuticle is treated with water at 100° C. for several days or, better, the chitin purified or further changed to chitosan, a rather coarsely fibrous structure is revealed. These fibers, commonly called Balken, average about 0.5 μ in diameter (range 0.3–1.0 μ), have a more variable spacing which, however, roughly approximates the fiber diameter, and are strongly birefringent with sharp extinctions (Fig. 7). As shown in Figure 1, where for clarity of the diagram the fiber diameters are reduced and the spacings between fibers exaggerated, these fibers occur in layers (Balkenlagen) which apparently correspond to laminae of the cuticle. Four orientations can be recognized forming a crossed-fiber type of orientation: one is longitudinal, one transverse, and two oblique at roughly a 40° angle with the longitudinal set. It was not found possible to count the number of superimposed Balken but since sections are similarly birefringent from outer to inner surface (Fig. 8) the number may well be the same as the total number of laminae. Although four orientations could be readily recognized, the sequence of these was not determined because of technical diffi-

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Magnification of individual photographs indicated by a 10 μ line on each picture.

FIGURE 6. Free-hand tangential section of an antenna fixed in Bensley's fluid and examined unstained in water. Shows distribution of sense plates (large ovals), sense hairs (smaller circles) and pore canals (very small spots).

FIGURE 7. Free-hand tangential section of an antennal cuticle purified to chitosan with hot conc. KOH. Photographed between crossed Nicol prisms with a rotating compensator set to accentuate birefringence along one diagonal while reducing it along the other. Two crossed sets of diagonal Balken are shown. Compare with Figures 1 and 6.

FIGURE 8. Longitudinal section of an antennal cuticle purified for chitin in a 10% NaOH solution at 100° C. Photographed between crossed Nicol prisms.

FIGURE 9. Longitudinal section of an antenna fixed in Bensley's fluid and examined unstained in water. Photographed between crossed Nicol prisms.

FIGURE 10. Long and short sunken setae in a longitudinal section of an antenna fixed in Bensley's fluid and examined unstained in water.

FIGURE 11. Same photographed between crossed Nicol prisms showing the strong, sharply extinguishable, birefringence of socket walls and shaft.

FIGURE 12. Longitudinal section of an antenna that had been abraded with emery powder and then soaked in ammoniacal silver nitrate solution. The thinner parts show precipitation of silver in the pore canals between cuticular laminae.

FIGURE 13. Longitudinal section of an antenna which had been frozen at − 195° C., dried by sublimation at − 35° C., and then embedded in paraffin *in vacuo*. Stained with Mallory's triple stain. Arrow shows area where the epicuticle is separated from the surface of a sense plate.

FIGURE 14. Longitudinal section of an antenna fixed in cold Bouin's fluid and stained with Heidenhain's haematoxylin. Shows differentiation of the sense plate socket and of the epicuticle.

FIGURE 15. Another photograph from the same section but from the region where sense hairs but no sense plates are found.

FIGURE 16. Section of a pupal antenna stained with Mallory's triple stain and photographed with a green filter. Shows the future membrane between antennal segments, and developing sclerotization at each end of this membrane.

FIGURE 17. Section of the membrane between antennal segments of a teneral adult stained with the periodic acid-Schiff reagent method for polysaccharides. Epicuticle intense red; endocuticle faint pink.

FIGURE 18. Similar to preceding but from a late pupa. Epicuticle intense red; procuticle and epidermal cells light pink.

culties arising from the fact that after alkali treatment one set of fibers droops into the interstices of the next. It can be recorded that in all of the several dozen specimens examined, the outermost layers of fibers were oblique.

At least the outermost sets of Balken fuse into a rim around the sockets of setae and other sensilla. Presumably lower layers do likewise. Here and there a Balken fiber can clearly be seen to branch but without any constant angle being involved; some gradually separate at a small angle, others branch off almost at a right angle and then turn abruptly to become parallel.

There seem to be two general possibilities for the origin of Balken fibers: either they are giant micelles or they are micellar aggregates (Richards, 1951). Branched Balken of diverse diameters, illustrated by Langner (1937) for diplopod cuticles, favor the idea of micellar aggregates (also, Biedermann (1903) reported that Balken seem to be composed of more minute fibers). This is strongly supported for the Balken of honeybee antennae by the fact that electron micrographs of the frayed edges of antennal sections (Fig. 28) sometimes show Balken separated into smaller fibrils that extend down to micellar dimensions (Richards and Korda, 1948). Further support for the idea that Balken are micellar aggregates comes from the purely chemical separation of "submicroscopic Balken" of sense plates into fibrils of micellar dimensions (Figs. 27, 29). These data are consistent with Kühnelt's (1928) suggestion that Balkenlagen are only specialized cuticular laminae. No evidence was obtained as to why successive laminae show different orientations and so produce the crossed-fiber type of structure.

The Balken sets extend around the antennal segment and appear entirely similar in the bands with and without sense plates. However, at the ends of each antennal segment the Balken lose their identity in the basal and apical rings. These areas appear to lack a Balken type structure, or, if one prefers, to be a single larger Balken (since they are an indivisible band of tightly packed, paralleled molecular chains). These apical rings show sharp extinction with the chitin micelles extending circumferentially (= tangentially) around the antennal segment.

The author was somewhat surprised to find the optical effects showing that the soft intersegmental membranes of the antennae have closely paralleled micelles. In this case there seems to be no tendency for the micelles to aggregate into Balken —at least the membrane appears uniformly birefringent. The sharp extinction shows that this membrane does not have a crossed-fiber type of structure; the micelles are parallel to the axis of the antenna and hence at a 90° angle to those they join at the apical and basal ends of each segment.

Longitudinal sections show a laminate structure between crossed Nicols due to different amplitudes of birefringence in successive laminae (Fig. 8). A similar picture from tick cuticle has been published by Schulze (1932). Such an effect is to be expected from any single plane through sets of crossed fibers even though each lamina would be equally birefringent if properly oriented.

The size and spacing of the relatively large pore canals (Fig. 6) is consistent with their passing between Balken and through the interstices of crossed Balken sets.

Micelle Orientations in Setae

The cuticle of setae ("sense hairs") and setal sockets, unlike the general surface cuticle, is distinctly birefringent in its normal state (Figs. 9, 11). The explanation of this difference is not known although several suggestions are possible (see Richards, 1951, p. 84). The amplitude of setal birefringence can be increased by purification of the chitin.

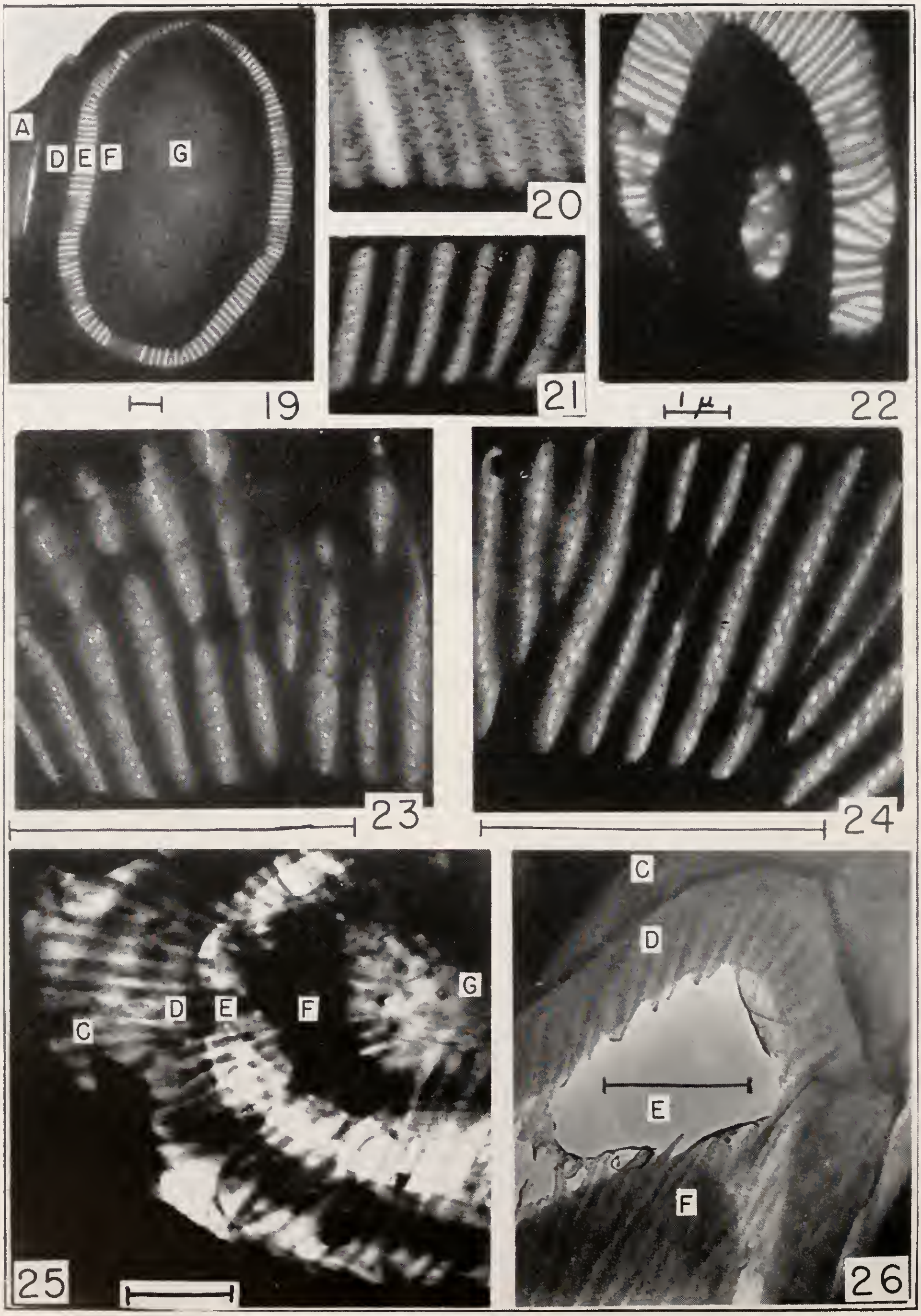
Both the sense hairs and their socket rims show sharp extinction indicating a high degree of orientation. The micelles are parallel to the setal axis along the shaft, radial in the thin soft socket membrane, and circumferential or tangential in the socket rim. The shaft of sense pegs is similar but the sockets show less birefringence (details of which were not determined). Both long and short sunken setae (Figs. 10–11) show orientations similar to those of tactile setae; since the tormogen membrane is, in effect, stretched and elongated to form the setal pocket, the radial micelles of this membrane are also oriented approximately parallel to those of the setal shaft.

Clearly, micelle orientations do not depend on sclerotization for stability because equally sharp extinctions are given by soft membranes (staining blue with Mallory's stain), by incompletely sclerotized membranes (red with Mallory's stain), and by heavily sclerotized membranes (refractory to stains). The extensive analysis of *Drosophila* setae by Lees and Picken (1945) suggests that growth pressures produce the longitudinal orientation in setal walls while they are being laid down.

The General Structure and Micelle Orientations of Sense Plates

The sense plates, or cuticles thereof, are thin complicated membranes overlying a group of cells that project most of the way through the cuticle (Figs. 33, 46). In surface view (Figs. 1, 6, 19) they are seen to be more or less oval structures whose long axes tend to parallel the axis of the antenna. A central plate (Fig. 1, *F–G*) is surrounded by a very thin membrane (*E*) which joins a thick rim (*D*) which in turn is surrounded by a socket rim of the general cuticle (*A*) formed by the fusion of Balken. The sensillum attains this recognizable differentiation very early in cuticle development (Figs. 38–39) but the following notes are based entirely on the condition found in the fully formed organ on adult antennae. Some authors have suggested that these peculiar structures can be derived, in a phylogenetic sense, from setae by assuming that the setal shaft is flattened down to become the central plate (Snodgrass, 1925).

In cross or longitudinal section (Fig. 2), it is seen that a double-layered epicuticle extends continuously across the sense plate (*D–G*) and adjacent cuticle (*A*). Beneath the epicuticle is a continuous chitin-protein cuticle. This is only partially sclerotized in the sense plate and its rim (*C–G*) but heavily sclerotized in the surrounding cuticle (*A–B*). If the epicuticle is removed or disrupted and the sense plate caused to shrink under electron bombardment, the structure straightens out in the manner diagrammed in Figure 3. There is no question that the rim is firmly attached to the cuticle at *B–C* (this can occasionally be seen in stained sections examined with the light microscope too), but it is not certain whether the chitin micelles fuse into those of the rim at this point or extend inwardly to form a lining of the cavity which contains the cells of the sensillum. It is also uncertain whether the space diagrammed between *A* and *C–D* is a real space or only a line of weak linkages; it does, however, coincide with a line of differentiation demonstrable with either Mallory's or Heidenhain's stains (Fig. 14).


A
D
E
F
G
19
20
21
1 μ
22
23
24
C
D
E
F
G
25
C
D
E
F
26

FIGURES 19–26

Examination with an electron microscope reveals that the thin membrane is traversed by some 120–150 radially arranged thickenings (Fig. 19). Over 80% of these are simple rods, well over 90% are either simple or bifurcated rods, but a small percentage of triply, quadruply or irregularly branched ones are seen (Fig. 5). Examination of extracted specimens shows that these thickenings are rod-like structures that may be compared to the Balken of the general antennal cuticle from which they appear to differ only in being beyond the limits of resolution by visible light microscopy. These "submicroscopic Balken" extend centrally into the middle of the sense plate (Fig. 1) and then lose their radial orientation (Fig. 22); they also extend through the rim *D* and on at least to *C* maintaining their radial orientation (Figs. 25–26). By prolonged and rather violent chemical treatments these Balken can be broken into microfibers of micellar dimensions (Fig. 29) and "stained" with zirconium or titanium to increase their contrast (Fig. 27).

In addition to the radially arranged micelles aggregated into Balken, there are circumferential (= tangential) micelles at least in areas *D* and *E*. For the rim *D* this is shown by polarized light effects. Figure 7 shows that the rims are birefringent in surface view; Figure 9 shows that the rims are also birefringent along radii of the sense plates but isotropic where the rim is cut in cross section. Accordingly, since the optic axis of chitin micelles is known to parallel the fiber axis (Richards, 1951), the micelles must be circumferential. Electron micrographs not included among the illustrations for the present paper indicate that a single layer of circumferential micelles extends around the membrane *E* and across the radially arranged Balken (specimens treated with iridium tetrachloride). It seems highly probable that the rows of holes seen in the membrane between Balken in Figures 21, 23 and

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Magnification of individual electron micrographs indicated by a 1 μ line on most of the pictures.

FIGURE 19. Relatively low power micrograph showing structure of the cuticle of a sense plate from a preparation cleaned in distilled water at room temperature (= normal). Lettering as in Figures 1–4.

FIGURE 20. Micrograph of a small portion of the thin membrane area (region *E* of Figs. 1–4 and 19) after soaking in ammoniacal silver nitrate solution (2 days). The dark spots represent silver deposits of the order of 100–200 Å. Magnification approximately equal to that of Figure 24.

FIGURE 21. A similar area of thin membrane after soaking in a 5% solution of phosphotungstic acid or $H_3PW_{12}O_{40}$ (1 day). The light spots appear to be breaks in the epicuticle between circumferential micelles (see text). Magnification as for preceding.

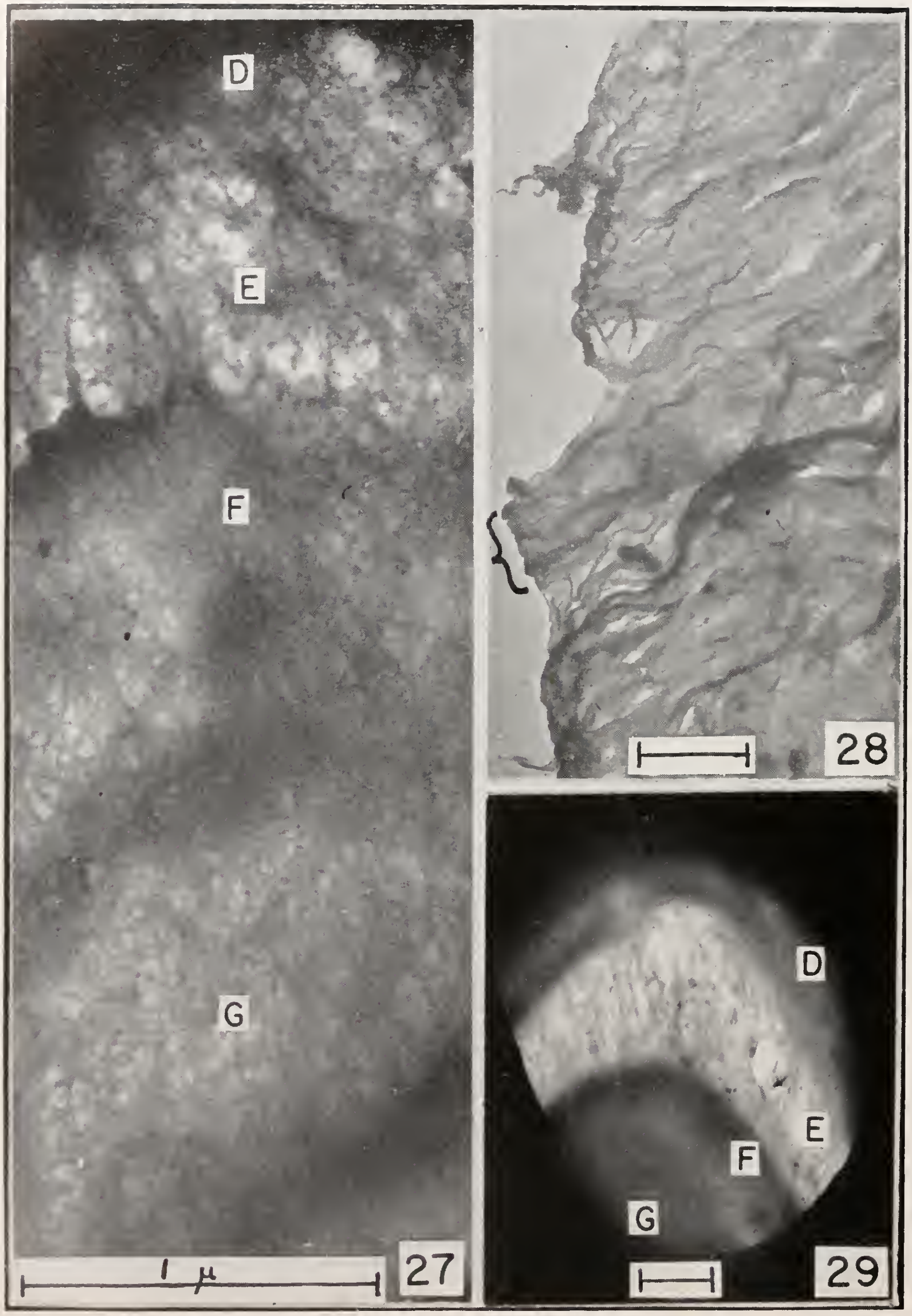
FIGURE 22. Another relatively low power micrograph of the cuticle of a sense plate after extraction in distilled water at 100° C. (1 day). Shows dispersion of a central area of the plate revealing thickenings.

FIGURE 23. A small portion of the thin membrane after soaking in a 2% solution of osmic acid or OsO_4 (1 day). The light spots are similar to those in Figure 21 (see text).

FIGURE 24. A small portion of the thin membrane after soaking in Diaphanol (3 days) following initial cleaning in distilled water and refluxing with chloroform.

FIGURE 25. One end of a sense plate after soaking in 30% H_2O_2 (7 days). The field of view represents the areas from *C* to *G* of Figures 2–4. Note the continuity of the thickenings (Balken) from *C* through *F*.

FIGURE 26. One end of a sense plate after soaking in Diaphanol (8 weeks). Shrinkage and electron destruction is obvious but note the cross striations on and between the radial thickenings.


D
E
F
G
1 μ
27
28
D
E
F
G
29

Figures 27–29

24 represent spaces between such circumferential micelles,[4] and that the cross striations between radial thickenings in Figure 26 do likewise.

A composite survey of the data on micelle orientations is given in Figure 4. Recapitulating, evidence for tangential orientation in the thick rim *D* is based on optical effects; radial orientation in *E–F* is based on both optical effects and electron micrographs; all other orientations indicated are based entirely on electron microscope pictures.

Dimensions of the various portions of sense plates are: the overall surface size averages 10 × 14 μ; the central plate averages 7.5 × 11 μ, and is 0.5–1.25 μ thick at the center (*G*) and 1–2 μ thick at the edge (*F*); the thin circumferential membrane normally appears to be about 0.6 μ broad in surface view but displaced sensilla and many electron microscope pictures show it as 1–1.5 μ broad (apparently due to the membrane actually extending part way across the rim *D*). The Balken in this membrane average 0.1 μ in diameter when dry with a range of 0.08–0.15 μ except for a few branched ones where the common trunk is as large as 0.2 μ. The membrane between these Balken (excluding the epicuticle which is several tenths of a micron thick) is only a few hundred Ångstrom units thick when dry; how thick it may be in the living insect is unknown but it can scarcely be more than a few tenths of a micron.

THE EPICUTICLE AND ITS DEVELOPMENT

The epicuticle forms a continuous, slightly birefringent outer sheath over the antenna, but being only a fraction of a micron thick, it is difficult to study. Its continuity without interruption across the sense plate from the surrounding cuticle (Fig. 2) is shown by certain slides, prepared by the freeze-drying technique, in which the epicuticle became separated from the underlying chitin-protein layer at some places (Fig. 13); continuity is also indicated by certain broken preparations which, when examined in the electron microscope, show a thin layer that seems certainly epicuticle projecting without apparent discontinuity from the sense plate, the surrounding cuticle and the minute intervening space. Apparent continuity can also be observed in ordinary stained sections viewed with a light microscope (Fig.

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[4] In Figures 21 and 23 the epicuticle has apparently broken under electron bombardment where most strained and least supported, *i.e.*, between the circumferential micelles; in Figure 24 the epicuticle has been removed chemically. These spaces are illustrative of one of the most serious technical difficulties involved in electron microscopy of insect cuticle, namely, that cuticle (and chitin) shrinks a considerable amount under electron bombardment (and may be readily "burned up" or distorted beyond recognition if the electron beam is made intense).

Magnification of individual electron micrographs indicated by a 1 μ line on each picture.

FIGURE 27. Portion of a sense plate after treatment, successively, in dist. H_2O, 30% H_2O_2 (9 days), 60% KOH at 160° C. (20 min.), and alcoholic 5% $TiCl_4$ (30 min.). Titanium reduced onto exposed amine groups acts as an electron stain to increase the clarity of the micelles. Lettering as in Figures 1–4 and 25–26.

FIGURE 28. Frayed end of a section of sclerotized cuticle between sense plates after treatment with 10% NaOH at 100° C. (1 day). The bracket indicates a single Balken visible with the light microscope.

FIGURE 29. One end of a sense plate after soaking in 60% KOH at room temperature (3 days) and then heating to 160° C. (15 min.). Shows separation of the radial thickenings into smaller fibrils of somewhat variable width, and, in comparison with Figure 27, low contrast correlated with the absence of an electron stain.

14) but, as Blower (1951) has emphasized, diffraction effects along the cuticle edge may create serious illusions in work on the epicuticle.

The epicuticle was not actually separated into two layers by any of the methods employed but it appears nonetheless double, at least in the sense that the outer layer or outer surface is stainable with osmic acid and Heidenhain's haematoxylin (Figs. 14–15). In comparison to the epicuticle over the general surface of the antenna, the epicuticle seems to be thicker on the intersegmental membranes (Figs. 16–18) and thinner across the sense plates but the thicknesses are too small for accurate measurement in these sections.

Data on the stainability of the epicuticle of various antennal areas at several developmental stages are summarized in Table II. In very early stages of development in the pupa it is extremely difficult to differentiate between the epicuticle and a possible precipitate of the molting fluid on the surface (Figs. 35–38). Mallory's stain colors both epicuticle and molting fluid blue on the future sclerotized areas and gives differentiation only at the future intersegmental region where the epicuticle is colored red, but the Schiff polysaccharide test (Fig. 18) fails to stain the precipitated molting fluid. During these early stages there appears to be a definite membrane (blue with Mallory's stain) outside the developing adult cuticle (Figs. 36–38). The nature of this membrane remains to be studied but the fact that developing sense hairs and sense pegs seem to push into it or push it outward suggest that it is not a part of the forming adult cuticle. Perhaps it is the same as an "ecdysial membrane" found by C. M. Williams and V. Passoneau (unpublished) in *Cecropia* pupae—they consider it to be an undigested innermost lamina of the pupal cuticle.

TABLE II

Changes in staining reactions of the epicuticle during development as seen with the light microscope. Upper term refers to outer layer (when distinguishable), lower term to inner layer of epicuticle; dash indicates refractory to staining by the indicated method

	Violet eyes, colorless antennae =Figure 36			Purple eyes, yellowish antennae =Figure 39			Teneral adult			Older adult		
	I.M.	S.P.	Scl.	I.M.	S.P.	Scl.	I.M.	S.P.	Scl.	I.M.	S.P.	Scl.
Schiff polysaccharide test	Red	Red	Red	Red Pink	Red Pink	Red Pink	Red Pink	Pink —	— —	Red Pink	— —	— —
Argentaffin test				Spots? —	Spots? —	Spots? —	— —	— —	Gray —	— —	— —	Gray —
Osmic acid 2%				— —	— —	— —	— —	— —	Black —	— —	— —	Black —
Mallory's triple stain	Red	Blue	Blue	Red Red	Red ?	Red —	Red Pink	Purple —	— —	Red Pink	Purplish —	— —
Heidenhain's iron haematoxylin				Brown Black	Brown Black	Brown Black	Gray —	Black —	Gray —	— —	Gray —	Black —

I.M. = intersegmental membrane between antennal segments.
S.P. = sense plates on antennae.
Scl. = sclerotized general surface of antennae.

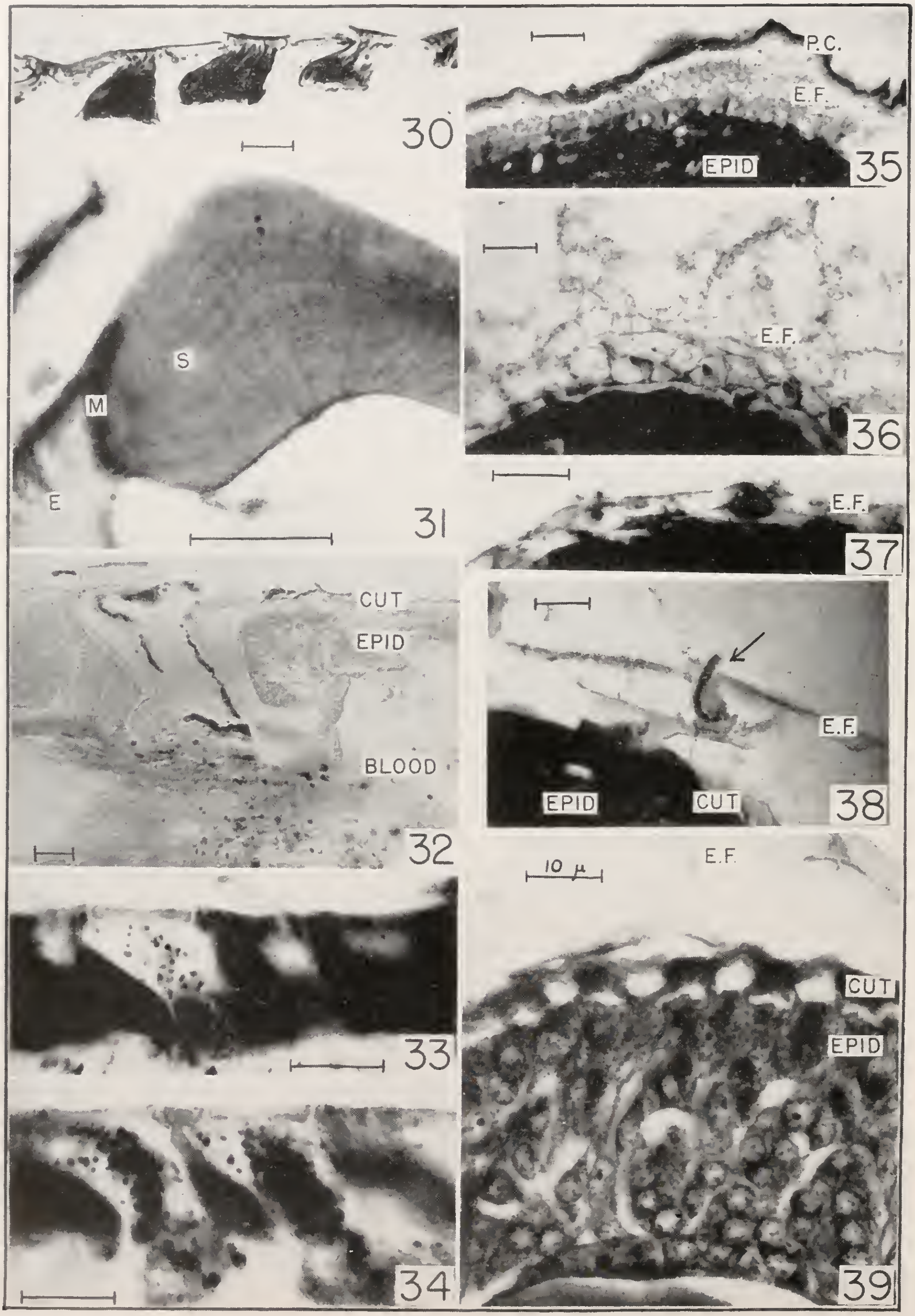
There is even more uncertainty about the interpretation of staining obtained with the argentaffin reaction; clearly the surface precipitate in Figure 32 is considerably thicker than the entire epicuticle, and clearly the silver deposits revealed by electron microscopy are more restricted than the sclerotization effect (Fig. 20 and Richards, 1951, Fig. 64B)—the data make one wonder about the degree of localization obtainable with the argentaffin reaction but we lack detailed knowledge of the chemical reactions involved.

Data presented in Table II show that the epicuticle at first stains uniformly over the antenna. An early differentiation giving different isoelectric points in different areas is implied by the results with Mallory's stain. Subsequently, differentiation results in the ability to recognize at least three types found respectively on the intersegmental membrane, the general sclerite surface, and the sense plates (Richards, 1952b). With Mallory's stain, Heidenhain's haematoxylin and the Schiff polysaccharide test, the change with time is due to partial or complete masking of the reactions given by early stages; with the argentaffin and osmium tests, the change is due to initiation of a positive reaction.

The precipitation of osmium from a 2% solution of osmic acid is detectable with the light microscope only on the general sclerite surface. However, use of the electron microscope reveals a general but very light deposit across the sense plate membranes (Fig. 23). This osmium reaction is prevented when the preparation is previously extracted with hot chloroform. It follows that a very small amount of lipid is to be found in the epicuticle across sense plates. Comparison of osmium-treated preparations with chloroform-extracted ones failed to reveal any recognizable lipid or wax layer, but it should be remembered that the electron microscope studies were limited to surface views where superimposed layers can be distinguished with certainty only when one layer ruptures and peels away from the other.

It has already been mentioned in a previous section that the epicuticle of the thin membrane over sense plates may show rows of minute holes (50–300 Å) after certain treatments (Figs. 21, 23). No such perforations are to be seen in normal preparations with nearly the same resolution. It is thought by the author that these holes in treated epicuticles are produced by the destructive effect of electron bombardment at points of lesser support by underlying circumferential micelles in the chitin-protein layer. Similar holes are to be found in preparations from which the epicuticle has been removed (Fig. 24). There is nothing to indicate that such holes are to be found in normal membranes.

The recognizable subdivisions of the epicuticle have been referred to by the noncommital terms "outer" and "inner." The inner subdivision develops a natural amber color and early becomes resistant to staining; it apparently is the same as the "cuticulin layer" of Wigglesworth (1947, 1948) and the "protein epicuticle" of Dennell (1946). No pore canals (readily stained in the procuticle) could be traced into this layer at any of the stages examined. The outer subdivision is stainable with haematoxylin, osmic acid and ammoniacal silver nitrate; it accordingly is thought to contain protein, lipid and polyphenol but as far as could be determined these occur together, not in separate superimposed layers. The outer subdivision, then, compares with Dennell's "lipid epicuticle" and with Wigglesworth's "polyphenol layer" plus "wax layer." By definition, no tectocuticle (cement layer) can be present since no dermal glands are to be found (also, no layer was separated off the surface by heating in chloroform).


30
P.C.
E.F.
EPID
35
S
M
E
31
E.F.
36
E.F.
37
CUT
EPID
BLOOD
32
EPID
CUT
E.F.
38
10 μ
E.F.
33
CUT
EPID
34
39

Figures 30-39

The Chitin-Protein Cuticle and Its Development

When the adult cuticle begins to develop it is at first a single thin layer that represents the epicuticle (Fig. 36). Within the next few hours there is a rapid increase in thickness, involving the epicuticle becoming visibly double and initiation of production of the chitin-protein procuticle (Richards, 1951). Fortunately for the present study, the sense plates become recognizable very early and can be seen to have already differentiated into central plate, circumferential membrane and rim by the time the cuticle is 1–2 μ thick (Fig. 38). Further development to full thickness (ca. 15 μ) occurs within a day, and events leading to sclerotization commence while the procuticle is still increasing in thickness.

Before treating the unusual details of sclerotization in these antennae, mention should be made of a paper now in press by Schatz (1952) proposing a terminology that will be followed here. Studying sclerotization in various insects, using primarily the differential coloring obtained with Mallory's triple stain (supplemented by haematoxylin and the Millon reaction), Schatz concluded that differentiation of the original soft procuticle is at least descriptively a two-stage process, and that the differentiation results in three rather than two recognizable subdivisions of the chitin-protein portion of the cuticle. The original transparent procuticle stains blue with Mallory's; a portion of this, while remaining transparent, changes to staining red with Mallory's; a portion of this last subsequently becomes refractory to staining and simultaneously develops its own amber or brown color. Classically, the hard colored part is called "exocuticle," the softer, transparent part "endocuticle."

Magnification of individual photographs indicated by a 10 μ line on each picture.

Figure 30. Unstained longitudinal section of the cuticle from a split antenna extracted in dist. H_2O at 100° C. (2.5 days). Shows failure of the treatment to remove the pigmentation.

Figure 31. Longitudinal section through the swollen cuticle at the base of an antennal segment of an old adult (Mallory's triple stain). Shows swollen laminae made distinct by thin fuchsinophile lines, pore canals stained by fuchsin, and dark red band (*M*) separating sclerite (*S*) from the blue-staining membrane (*E*).

Figure 32. Longitudinal section of a pupal antenna that was removed from the pupal case, soaked in ammoniacal silver nitrate (1 hour), then fixed in Carnoy's fluid, sectioned and mounted without additional staining. Note the argentaffin positive granules on the surface of the cuticle and in the blood cavity within the antenna.

Figures 33–34. Longitudinal sections of the antenna of an old adult. The antenna was split longitudinally and then treated with ammoniacal silver nitrate in the same manner as the preceding. Shows dense aggregations of argentaffin-positive granules in the cells of the sense plates, and (less clearly) the absence of such from cells of the sense hairs and general epidermis.

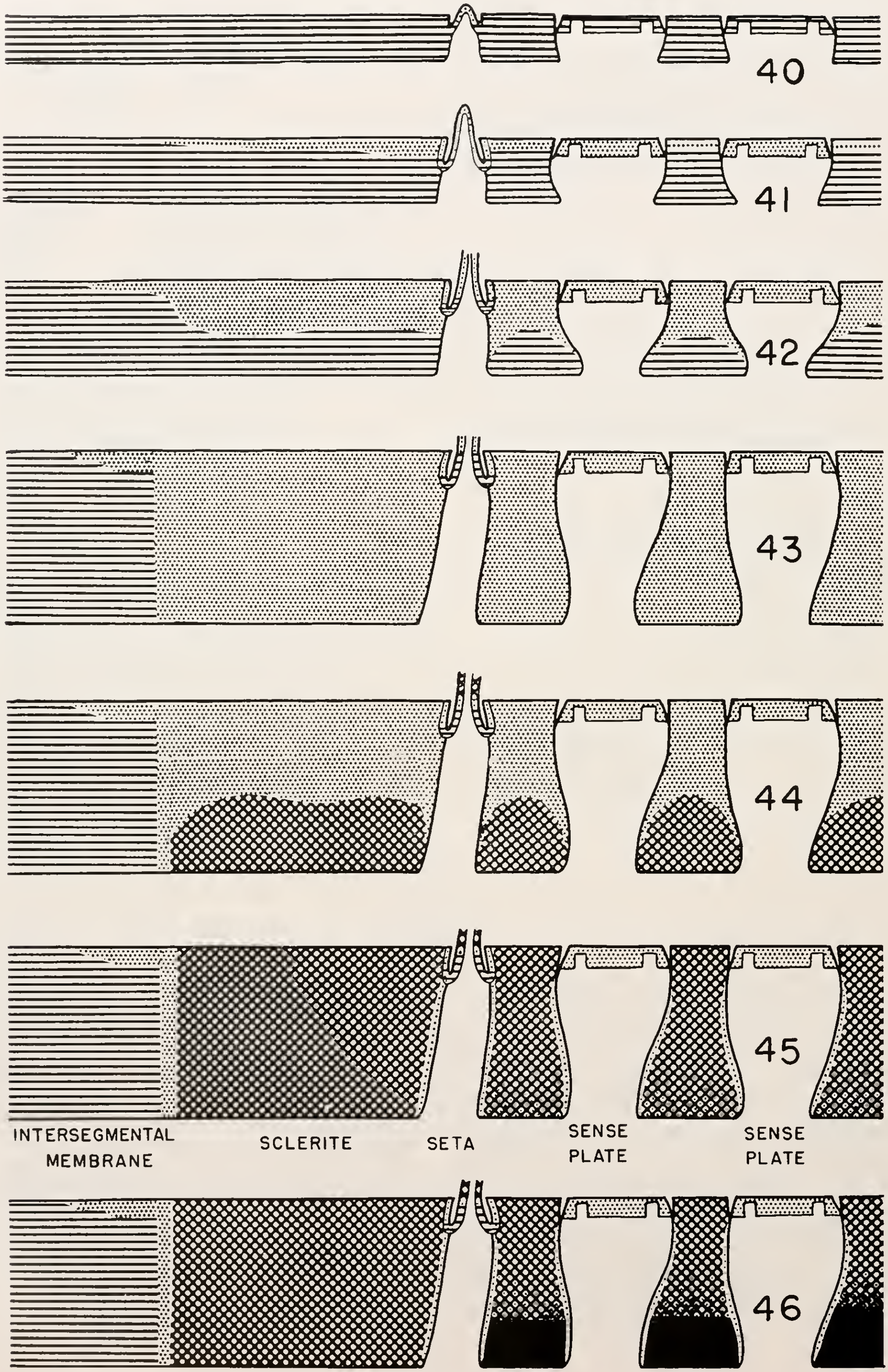
Figure 35. Cross section of the antenna of a pupa fixed in Carnoy's fluid, stained with Mallory's triple stain, and photographed with a green filter. This is an early stage in cuticle development; the print was made dark to show the precipitated molting fluid (stained blue) between the pupal cuticle (*P.C.*) and the epidermal cells (*EPID*).

Figure 36. A similarly prepared section from a slightly older pupa. Note developing sense hairs projecting into precipitating molting fluid (*E.F.*). Corresponds to an early part of the stage diagrammed in Figure 40.

Figure 37. Another similarly prepared section from a pupa of approximately the same age as the preceding. Note the distinct extra membrane.

Figure 38. A similarly prepared section from a slightly older pupa when the adult cuticle is 1–2 μ thick. Note the displaced half of a sense plate (arrow) which is lying in surface view on top of the extra membrane. Corresponds to a late part of the stage diagrammed in Figure 40.

Figure 39. A similarly prepared section from an older pupa when the cuticle (*CUT*) is nearly half the final thickness. Corresponds to Figure 42.


40
41
42
43
44
45
INTERSEGMENTAL MEMBRANE
SCLERITE
SETA
SENSE PLATE
SENSE PLATE
46

Figures 40–46

In a fully hardened cuticle, hard exocuticle is usually separated from blue-staining endocuticle by a red-staining line or band, and hard exocuticle goes through a red-staining stage during its development. Schatz proposes that the hard colored part be called exocuticle, the transparent but fuchsin-staining part be called mesocuticle, and the transparent and still blue-staining part be called endocuticle. Incidentally, the mesocuticle was the only one of these found to stain with Heidenhain's haematoxylin and with the Millon reagent. A somewhat similar picture has recently been presented by Ito (1951) and Blower (1951). Blower has proposed the term "prosclerotin" for the substance giving the Millon reaction. In the present case a purely descriptive histological term seems desirable, and mesocuticle will be used here without implying any particular chemical meaning.

Sclerotization of the bee antennae commences when the cuticle is $\frac{1}{4}$ to $\frac{1}{3}$ its eventual thickness, and follows the novel sequence diagrammed in Figures 40–46. At first the entire procuticle stains blue with Mallory's stain (Fig. 40). Then, beginning at the outer surface, a change commences (Fig. 41) and spreads inwardly (Figs. 16, 42–43) transforming all of the procuticle of future sclerotization areas into red-staining mesocuticle. The epicuticle is not altered in its staining reactions at this time, and still seems blue over future sclerites; setae are slightly in advance of the general cuticle; the procuticle of sense plates has reached its final staining condition by the stage shown in Figure 42 and will not change further; the future intersegmental membrane thickens without changing its staining reactions; and by the time this change of future sclerite areas to mesocuticle is complete, the cuticle has reached its full thickness. Then, and this is the novelty involved, *beginning at the inner surface and spreading outwardly* (Fig. 44), the mesocuticle becomes amber colored and refractory to staining in prospective sclerite areas, *i.e.*, becomes typical exocuticle throughout its entire thickness. Subsequent to completion of this sclerotization (Fig. 45) the inner $\frac{1}{3}$ to $\frac{1}{2}$ becomes increasingly brown and eventually quite dark (Fig. 46), but for some unknown reason this additional browning of the inner portion of the exocuticle occurs only in the exocuticle between sense plates—the exocuticle of the band free from sense plates remains unmodified.

Summarizing for the condition found in the chitin-protein portion of the cuticle of the fully developed adult (Fig. 46): The intersegmental membrane stains blue and is accordingly all endocuticle except near the sclerites where the outer portion is red-staining mesocuticle. The intersegmental membrane is separated from sclerite areas by a red line of mesocuticle (Fig. 31). The sclerotized areas are completely exocuticle except for thin red layers lining the setal and sense plate apertures, but the inner portion of areas between sense plates is modified by additional darkening. Sense hairs and sense pegs become all exocuticle except for the tormogen membrane, whereas sunken setae and sense plates remain in the mesocuticle stage and have a similarly staining tormogen membrane.

This sequence in sclerotization is readily rationalized if we assume a two-stage

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FIGURES 40–46. Diagrammatic presentation of the sequence of developmental stages of the procuticle of honeybee antennae as shown by preparations stained with Mallory's triple stain. Parallel lines = procuticle and, in later stages, endocuticle; stipple = mesocuticle; cross-hatch = exocuticle; solid black = exocuticle modified by additional dark pigment. See text for explanation.

process controlled by two agents (enzymes?), one of which is effectively located at the outer surface, the other at or beyond the inner surface. Assuming two such agents, there is no known reason why they might not have the reverse distribution to that recorded for these antennae (and this is indeed what Schatz (1952) records for the cockroach *Blatta orientalis*), or both be active from the outer surface (as seems to be generally true), or both be active from the inner surface (not yet recorded).

The distribution of granules reacting with ammoniacal silver nitrate has one interesting feature. In the earliest stages, the entire antenna is negative to the argentaffin test. Somewhat later, numerous positive granules are seen in the blood space and on the outer surface of the developing cuticle (Fig. 32). Still later, positive granules are found in the epidermal cells over the entire antenna. Then, as sclerotization is completed, these granules disappear from the epidermal cells except along the soft intersegmental membrane and in the cluster of cells under sense plates. In the adult, the cells under sense plates are loaded to various degrees with large argentophile granules (Figs. 33–34) whereas cells under the general sclerite surface and under sense hairs and sense pegs are free of such granules. The argentaffin reaction is only semispecific and correlation is not proof, but the data are consistent with the idea that such granules supply the polyphenol derivative used as substrate to change the mesocuticle to exocuticle, and that this change is controlled not by which cells are permeable to this substrate from the blood but by which cells pass it out into the cuticle. A cursory check of argentaffin reactions in developing cuticles of *Blatta, Oncopeltus, Phormia* and *Apis* thoraces suggests that this argentaffin sequence may be another peculiarity of honeybee antennae.

The diffuse dark color developed in the inner part of the exocuticle between sense plates is no staining effect. It can be seen in free-hand sections examined in water and in unstained frozen-dried preparations. It is only slightly if at all decreased in intensity by prolonged treatment with hot water (Fig. 30), a treatment which changes the cuticle from virtually isotropic to moderately birefringent. It is not visibly affected by Carnoy's fixing fluid or common solvents such as alcohol, acetone, chloroform, etc. Conceivably it might be a melanin formed from excess substrate not needed for sclerotization.

Incidentally, the fully hardened exocuticle of adult antennae is darkened or blackened by ammoniacal silver nitrate and 2% osmic acid. The darkening by OsO_4 is not prevented by previous refluxing with boiling chloroform and so must be due to some agent not removed by chloroform or destroyed by heating to 61° C.

The relatively large pore canals (Fig. 6) follow a straight or slightly wavy course from the epidermis to the epicuticle, penetrating between interstices of the crossed Balken. The pore canals are stained by ammoniacal silver nitrate, Heidenhain's haematoxylin and Mallory's stain (red); they are not stained by osmic acid or by the Schiff polysaccharide test. Even in the old adult, sections in which the epidermal cells are slightly separated from the cuticle show cytoplasmic strands extending to and seemingly into the pore canals, and electron micrographs of tangential sections which have been soaked in distilled water show the canals as holes. It follows that these canals never become plugged with cuticle. The only notable point is the curious observation that in regions thin enough to see through in sections stained with ammoniacal silver nitrate, rows of spots occur along the pore canals at intervals agreeing with the spacing of cuticular laminae. Similar precipitation of silver spots along pore canals of *Tenebrio* cuticles was obtained by Day (1949) using a technique thought by some to be specific for ascorbic acid.

Comparison of Various Sensillum Types

The sense hairs and sense pegs differ in cuticle thickness but the cuticles of both are fully sclerotized in the adult and appear completely negative to staining by Mallory's and Heidenhain's stains, 2% osmic acid and the Schiff polysaccharide test. The tormogen membrane of each of these consists of a ring of blue-staining endocuticle (Mallory's stain) separated from the exocuticle of the setal shaft by a ring of red-staining mesocuticle and connected to a similarly red-staining rim of mesocuticle.

The sense plates and sunken setae (both long and short) differ in that they stain red with Mallory's, black with Heidenhain's and pink with the Schiff polysaccharide test; as far as can be determined with the light microscope they are negative to osmic acid (see preceding section on sense plate epicuticle). These stain completely red with Mallory's, *i.e.*, unlike the sense hairs and sense pegs, they have no ring of blue-staining endocuticle.

In the pupa, the sense pegs become sclerotized first, sense hairs next, and sense plates and sunken setae last, but the time difference is not great and there is no reason to think it important since all reach whatever stage of sclerotization they are going to develop considerably prior to emergence of the adult. The real difference is that the hairs and pegs become completely sclerotized whereas the plates and sunken setae undergo only the first change (to mesocuticle). And since these changes occur early in relation to general sclerotization of the antennae, control must be somehow effected by the individual cells responsible for the formation of the various areas.

Incidentally, the tip of sunken setae may possibly be different from the shaft. At least in some slides, the tips of longer sunken setae show a minute spot of osmium deposit, or in Mallory's preparations, a corresponding blue spot. In some the tip even appears flared. Unfortunately the size of this spot at the tip of long sunken setae is close to the limits of vision with a light microscope.

The above notes comparing the various sensilla probably refer exclusively to the chitin-protein cuticle. Except for the sense plates (preceding section), details concerning the epicuticle could not be satisfactorily determined because of decreased visibility due to the cylindrical structure.

Comparison to Thoracic and Abdominal Cuticles

The preceding sections have dealt exclusively with the antennal cuticle. When this work was nearing completion, cursory examination was made to see if the novel features shown by antennae were peculiar to the antenna or just characteristic of honeybees. Only cuticles of teneral adult honeybees were examined.

The epicuticle of sclerites is negative to Heidenhain's haematoxylin. On the thorax it was negative to Mallory's stain and the Schiff polysaccharide test, but on the abdomen faint red staining was obtained on the tergites and stronger staining (especially with Mallory's) on the intersegmental membranes. It follows that at least some areal differentiation of the epicuticle is found on other parts of the body besides the antennae but details remain to be worked out carefully. This is to be expected from the report of such areal differentiation in various species of insects (Richards, 1952b).

Most attention was given to the sclerotization picture in the chitin-protein subdivision. This clearly is not the same as on the antennae. On the thorax an outer exocuticle consists of a thin brown layer underlain by a broader amber zone; beneath this is a rather broad mesocuticle, and beneath this a thin endocuticle. On the abdominal tergum, endocuticle is limited to the intersegmental membranes but the sclerites consist of a more or less broad layer of amber exocuticle underlain by a somewhat thinner layer of mesocuticle. Adjacent to the intersegmental membrane are areas with microtrichia where the entire procuticle has been changed but only to mesocuticle.

Sclerites on the thorax and abdomen, then, present the orthodox differentiation picture. The pupal cuticle also shows the orthodox exo-, meso-, endocuticle sequence from outside to inside in sclerotized areas. These data show that the sclerotization sequence diagrammed in Figures 40–46 is a peculiarity of the antennae in honeybees, and on the antennae is shown by only the adult cuticle.

SUMMARY

1. The epicuticle on honeybee antennae differentiates in correlation with sclerotization to give qualitatively different reactions on different parts.

2. The procuticle of the antennae shows a novel and unorthodox sequence of sclerotization involving two stages. The first stage begins at the outer surface and spreads inwardly, the second begins at the inner surface and spreads outwardly (Figs. 40–46). As a result the entire thickness of cuticle becomes sclerotized but still shows reducing power for osmic acid and ammoniacal silver nitrate. In contrast, the cuticle of thorax and abdomen shows the orthodox sequence of sclerotization proceeding from the outer surface inwardly.

3. Argentophile granules disappear during sclerotization from epidermal cells of areas that undergo sclerotization. Large numbers of argentaffin-positive granules remain in the cell cluster under the sense plates.

4. In the strip containing sense plates, the antennal cuticle develops a dark brown color in its inner half. This browning, which is additional to the usual sclerotization, is not found in the strip which is devoid of sense plates.

5. The cuticle over sense hairs and sense pegs becomes completely sclerotized; the cuticle over sense plates and sunken setae undergoes only the first of the two stages involved in sclerotization.

6. It follows that the cuticle over chemoreceptors cannot be assumed to have the same penetration properties as cuticle on other parts of the body. Also, different sensilla with cuticles of similar thickness cannot be assumed to have similar penetration properties.

7. The color of sclerotized antennal cuticle is not noticeably affected by prolonged treatment with hot water (100° C.) but lack of complete resistance to the treatment is shown by a change from near isotropic to readily recognizable birefringence.

8. The sense plates are differentiated early in the formation of the adult cuticle. They develop a complicated micelle pattern involving both radial and tangential orientations (Fig. 4). Other sensilla also differentiate early.

9. Balken are divisible into smaller fibrils of micellar dimensions. Viewed from the surface they form a crossed-fiber pattern on the antenna with four recognizable orientations: longitudinal, circumferential and two oblique (Fig. 1). At the ends of the segments these Balken fuse into rims which show completely circumferential orientation; the rims in turn join intersegmental membranes which show completely longitudinal orientation.

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THE DEVELOPMENTAL HISTORY OF AMAROECIUM CONSTELLATUM. III. METAMORPHOSIS

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A description of metamorphosis in the compound ascidian, *Amaroecium constellatum,* is presented as the third in a series of papers covering its complete life history. The paper satisfies two needs. First, it helps to fill a gap in information about a tunicate widely distributed and well represented along the eastern shore. Second, it serves as a frame of reference for the presentation of the results of an experimental study on metamorphosis which forms the subject of another paper.

The extensive studies of Brien (1929, 1930), Brien and Blanjean (1939) on the embryology and metamorphosis of European ascidians and the excellent synthesis of Berrill (Tunicata, 1950), which reviews his own investigations and those of the earlier workers, provide a comprehensive background for the understanding of the process of metamorphosis in general. Such a background precludes the introduction of unnecessary details where the processes in Amaroecium parallel those already described in the other forms.

Absence in the literature of the complete life history of any one American tunicate is justification in itself for the completion of the present study.

Materials and Methods

Amaroecium constellatum was collected in the vicinity of Woods Hole, Massachusetts. The colonies were kept in shallow dishes in running sea water and put into a dark room at night to prevent the shedding of tadpoles shortly after sunrise. About twenty minutes after removing the dishes into the light tadpoles were shed and collected into Syracuse watch glasses for study. They were studied in the living state but opacity of body necessitated extensive use of stained and cleared specimens in whole mounts and serial sections.

The fixative that gave the best results was Schaudinn's solution heated to 60° C. and poured quickly and generously over the tilted dishes to insure the greatest possible extension of the animals. The Feulgen technique used on whole mounts produced very clear specimens at all stages, even those of the early periods that remain opaque in other stains. The sections were stained with alum haematoxylin and triosin. The photomicrographs were taken with a Zeiss photomicrographic camera.

Observations

Fixation of the larva

The tadpoles swim vigorously for a few minutes after being released through the cloacal aperture of the colonies. They alternately swim and rest in short periods, being attracted first to the light side of the dish where they rest, then swimming back to the side of the dish opposite the source of light (Grave, 1921). They swim for a time varying from a few to thirty minutes. At the end of the swimming period they leave the rim of water in the larger dishes and attach themselves to the side or bottom of the dish. In the Syracuse watch glasses they attach to the bottom, to the sides or to the surface film. Those that are attached to the surface film are lost when the watch glasses are put into running sea water.

At the moment of fixation the three adhesive papillae expel their secretion in an explosive action, and the attachment effected by this cementing substance may be broken without impairing a subsequent attachment at some other place. Upon explosion of the adhesive secretion the test vesicles detach themselves from the epidermal ridge, their point of origin, and move out into the tunic where they begin to secrete additional tunicin. Within three minutes they are detached from the mantle and are distributed throughout the area of fixation. Withdrawal of the papillae from the surface and the additional tunic secreted between them produce a temporary scalloped appearance at the larval anterior end of the fixing ascidiozooid (Fig. 3, G). Since there is no addition to the test at its posterior half at this time the tunic flares broadly at its anterior half. The test vesicles rapidly lose their secretory capacity and wander out to the periphery where, eventually, they lose their identity. They function only during the period of fixation and they provide, with some questionable aid from the secretion of the adhesive papillae, a cementing substance in which the fixing larva can anchor itself during the establishment of its adult axis.

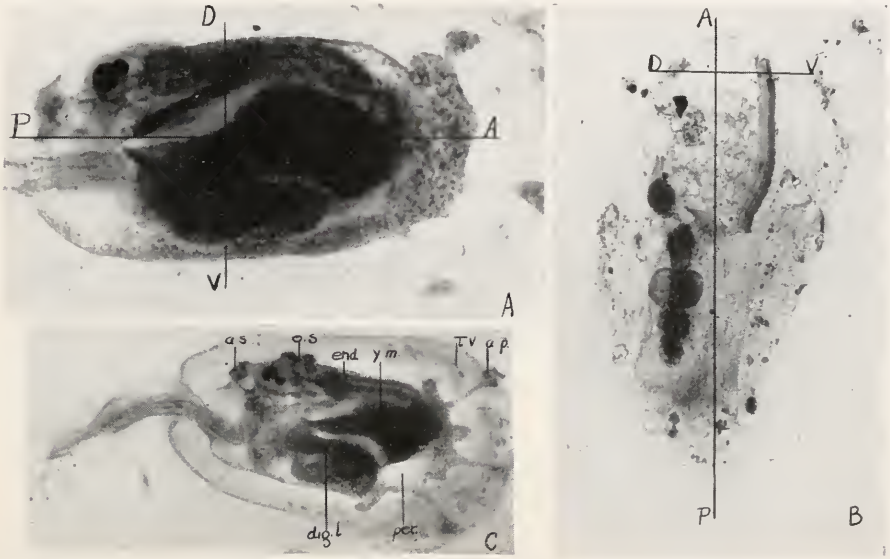

FIGURE 1. A. Tadpole at the moment of attachment showing larval axes. 200 ×. B. Ascidiozooid at the completion of metamorphosis, two days after fixation, showing adult axes. 50 ×. C. Tadpole, retraction of tail in progress. 125 ×. a.p., adhesive papillae; a.s., atrial siphon; end., endostyle; dig.l., digestive loop; o.s., oral or buccal siphon; per., pericardium; t.v., test vesicle; y.m., yolk mass.

The beginning of the fixation process is marked also by retraction of the axial structures in the tail. When the tadpole comes to rest for attachment the papillae explode their contents, the tail stops twitching and, in a quick turn, arches itself into a final bend (Fig. 1, C). Within two minutes the contents of the caudal envelope buckle into one or two folds without disrupting the linear relationship of the notochord, neural tube and muscle cells. Simultaneously, the axial structures of the tail begin to retreat with smooth, flowing action into the posterior clear area of the tadpole's trunk where they are drawn into a compact mass (Fig. 3). When the caudal contents are pouring into the trunk, the cuticle becomes crinkled, giving support to Berrill's opinion (Berrill, 1947) that shrinkage of epidermis due to nutritional exhaustion may be the mechanical force initiating tail resorption. Each kind of cell retains its histological integrity and relationship during the first day and a half of metamorphosis. There is no chemical change until the differentiating processes of metamorphosis are accomplished. The epidermal cells at the tip of the tail together with the caudal mesenchyme form a dense cellular knob which plugs up the point of entrance of the resorbed mass (Fig. 2, A). It gradually moves away from the surface of the tunic as the caudal organs contract into their final disposition in the trunk. The empty envelope of the tail hangs on to the young ascidiozooid for two or three days and then falls off.

TABLE I

Time schedule of rotation

Time in minutes	Tail activity	Caudal structures	Caudal sheath	Adhesive papillae	Axis of fixation	Test vesicles	Rotation
0	Contractions stop			Explosion of adhesive jelly			
1	Flexure	Buckling				Migration	
2		Beginning of resorption		Rounded knobs retreated from surface			
3			Crinkling			Distribution through area of attachment	Body contractions begun
6		End of retraction. Epidermal knob at surface	Empty sheath attached to body	Stalk detached at base or at cup by force of body contractions		Located at surface of flared tunic	
8–10		Epidermal knob within trunk			Siphonal area twisted into upright position on base of attachment	Loss of identity	Strong rhythmic contractions in progress

Withdrawal of the tail and concurrent activities of related parts can best be summarized in a chart with time schedule included (Table I).

In many cases the tail is not resorbed. It continues to hang on the body during metamorphosis executing occasional quivers up to the second and third day of development (Fig. 4, B). Action of the test vesicles and the initiation of rotation movements are the same whether the caudal elements are retracted or retained in the attached tail. Nothing in the larval structures of the tail influences the metamorphosic process. The enzymatic action required for autolysis or phagocytosis of the tail tissues is in no sense responsible for the activities that initiate, favor, or effect the shift in axis from larval to adult state or for the differentiation of the adult organization.

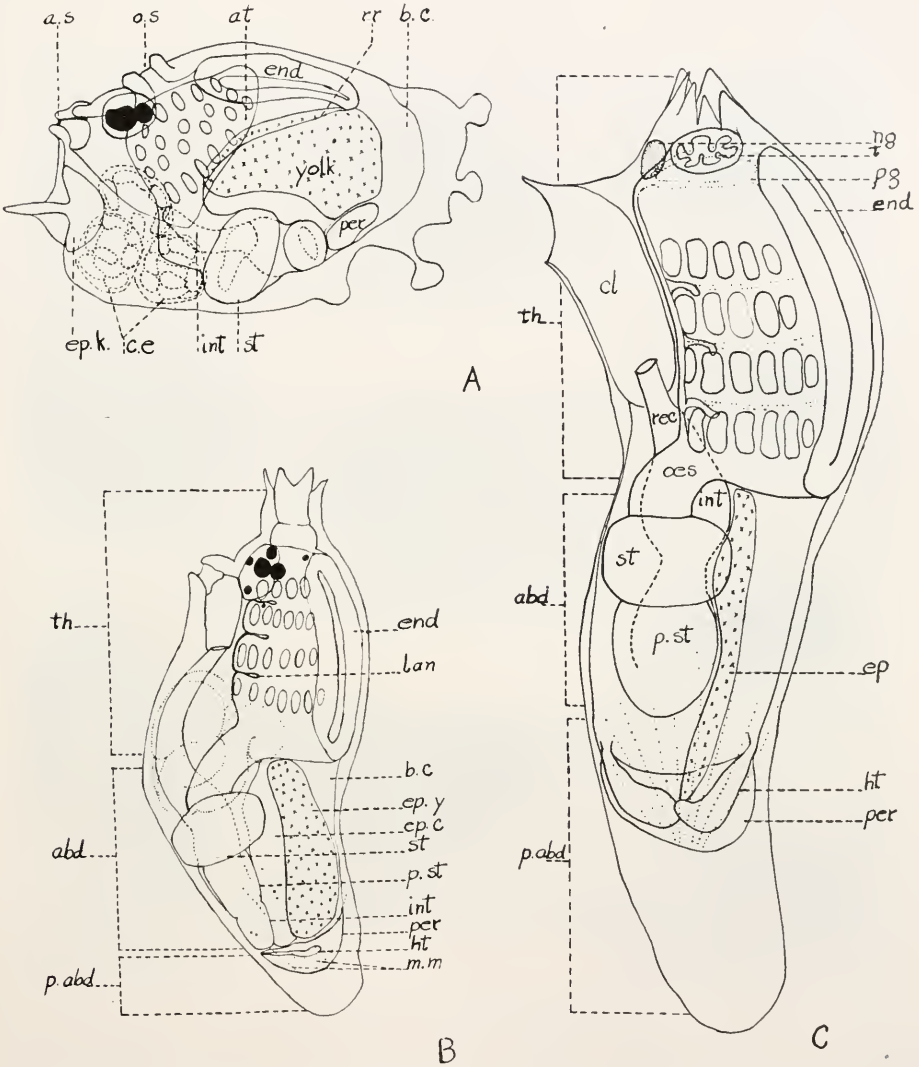
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FIGURE 2. A. Tadpole at the beginning of metamorphosis. B. Ascidiozooid, 12 hours after fixation. C. Ascidiozooid during the third day of development. Abd., abdomen; a.s., atrial siphon; at., atrium; b.c., body cavity; c.e., caudal elements; cl., cloacal chamber; end., endostyle; ep., epicardial tube; ep.c., epicardial cavity; ep.y., epicardial yolk; ep.k., epithelial knob; ht., heart; int., intestine; lan., languet; m.m., muscles of mantle; n.g., neural ganglion; oes., oesophagus; o.s., oral siphon; p.abd., post-abdomen; per., pericardium; p.g., peripharyngeal groove; p.st., post-stomach; rec., rectum; r.r., retropharyngeal raphe; st., stomach; th., thorax. (*Camera lucida* drawings.)

Rotation

For clarification of terms used in ascidian metamorphosis it should be recalled that the antero-posterior axis of an Amaroecium larva is marked by the adhesive papillae at its anterior limit and base of the tail at its posterior limit; dorsal refers to the area of the siphons and ventral to the region where the digestive loop is located (Figs. 1A, 2A). The antero-posterior axis of the adult Aplousobranchiata extends from the free siphonal area to the attached basal point (Figs. 1B; 2B, C). The pericardium is lodged in the basal tip of the post-abdomen. In the process of reorientation the yolk organ is projected into the lengthened post-abdomen and

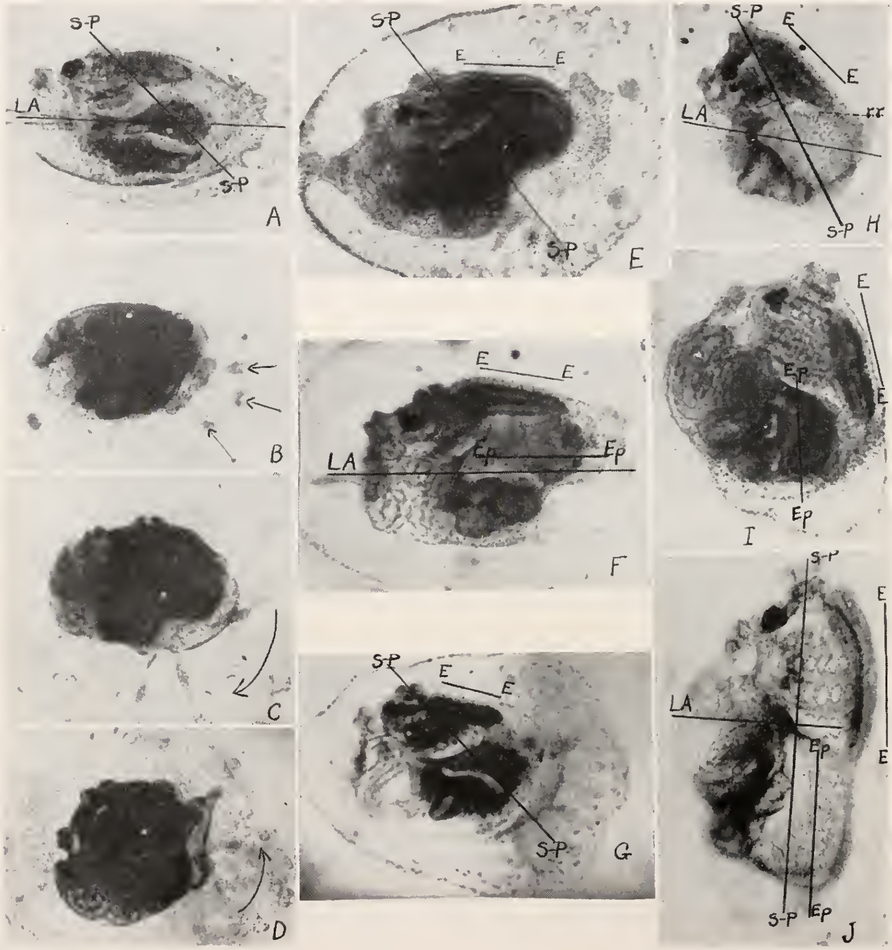

FIGURE 3. Series of photomicrographs showing body contractions of tadpole and rotation into adult axis. A, B, C, D, E, within 3 minutes of attachment. Arrows show direction of movement. F and G, 8 minutes; H, ½ hour; I, 2 hours; J, 3 hours after attachment. E–E, endostylar axis; Ep.–Ep., epicardial axis; La., larval axis; S–P, siphonal-pericardial axis; r.r., retropharyngeal raphe. About 100 ×.

the pericardium becomes separated from the digestive loop without changing their original topographical relationship (Fig. 4D). It is clear, then, that the antero-posterior axis of the adult corresponds with the dorso-ventral axis of the larva whereas the larval antero-posterior axis is temporary. The dorsal region of the adult is associated with the position of the ganglion and, therefore, all organs on that side are referred to as being dorsal; ventral is associated with the endostyle and all organs on that side are referred to as being ventral. An examination of Figures 1C and 2A will afford adequate review of the structure of the free-swimming tadpole and relationships of the larval organs to provide a basis for the presentation of metamorphic activities.

The process of rotation begins from one to eight minutes after the explosion of the adhesive jelly and the detachment of the test vesicles. It is effected by a series of contractions by means of which the organs are rotated through an arc of about 110°.

A strong contraction moves around the anterior end of the larva from the tip of the endostyle to the base of the tail on the ventral side, involving all the structures in that region of the body. The force of the movement pulls the stalks of the papillae into a sharp curve, in most cases detaching the entire papilla from the surface and reducing it to a knob-like projection of the body wall (Fig. 3A, B, C, D); in other cases jerking the stalk away from the secretory cup which is left temporarily at the surface. At the height of contraction the knobs or stalks protrude from the ventral body wall (Fig. 3C). The digestive loop is telescoped into an irregularly spiraled mass pressed against the caudal elements; the endostyle and yolk organ are shifted from the position of forming a straight angle with the siphons, coinciding with the larval axis, toward the direction of forming a right angle with the siphons and coinciding with the adult axis (Fig. 3). The endostyle may be thrown into several deep folds in keeping with the general state of contraction in all the viscera. In the living animal nothing of its anatomy is discernible when the contraction is at its maximum.

The reverse action toward the dorsal side follows immediately upon the preceding movement but it is less forceful, the organs not recovering their former alignment. Movements, once begun, alternate rhythmically through a period varying from 6 to 12 minutes, the ventral contraction always being the more violent. After that period the contractions are less frequent and less marked, each one effecting a change in the direction of the adult axis. Since the siphons and caudal mass do not participate actively in the contractions and remain unchanged in actual position and in relationship to each other in the course of the movements, it is possible to consider the siphonal area as a fixed point with reference to the reorientation of the axis. The angle of total body rotation is about 70° from larval to adult axis; the endostyle moves through an arc of 90° to 100°; the pericardium and yolk organ through 90° (Fig. 3).

The adult axis is established within two hours or less after the first contractions begin. The activities that accompany the forceful movements of rotation are processes of growth and extension, the former being marked by numerous mitoses evident in the endostyle, digestive tract and cells of the body cavity. The siphons become more elevated and prominent, the oral siphon developing six blunt lobes (Fig. 4C). Both openings are filled with primary tunic and are non-functional. The tight coil of caudal elements bulges from the surface on the morphological dorsal

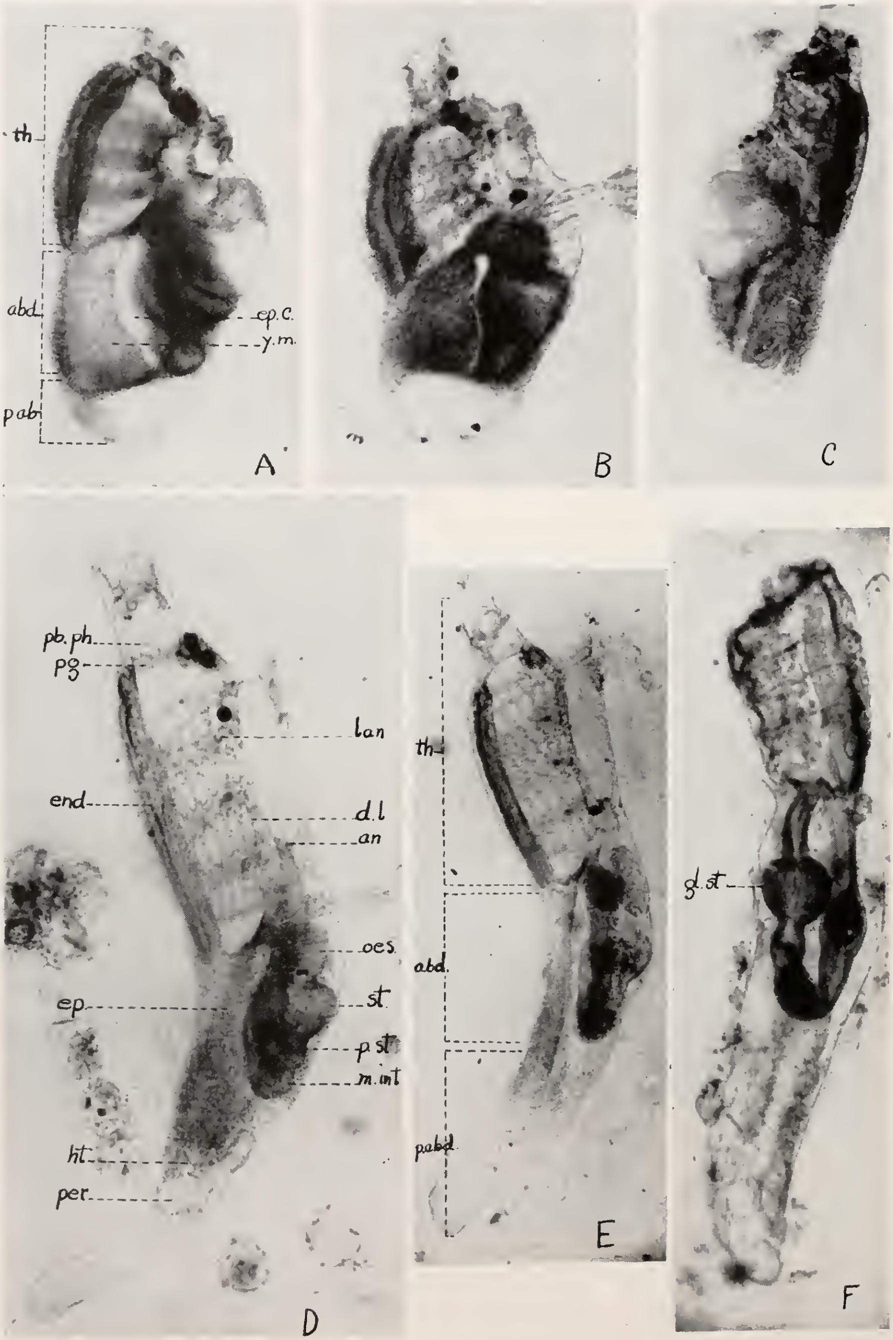
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Figure 4

side with the intestine pressed against it medially (Fig. 4A). The organism appears short and compact during the early stages of metamorphosis and becomes progressively more transparent as reorganization proceeds.

When rotation begins, the pharynx is limited in size, the peripharyngeal cavities pressing into it laterally and the nutritive endoderm occupying most of its space ventrally (Fig. 5A, B, C). With each movement of rotation the pharynx elongates in the direction of the developing axis and the peripharyngeal cavities, correspondingly, stretch into narrow chambers clasping right and left sides of the pharynx and meeting in the common cloacal cavity at the atrial siphon. The four rows of gill clefts come into evident view as the thorax elongates (Fig. 3G, I).[1]

Expansion of the pharynx is accomplished both by extension in length in the adult axis and by gradual expulsion of the yolk organ from its location in the floor of the cavity. The yolk organ is the mass of nutritive endoderm derived from the vegetal cells. It forms two thick-walled longitudinal furrows with an extension of the pharyngeal cavity below the yolk mass (Fig. 5G). The cavity is evident as a space between digestive loop and yolk in lateral views of the tadpole. In such views the whole mass appears wedge-shaped, its broad base facing the adhesive papillae, its narrow apex pointing toward the root of the tail (Figs. 1C, 3G).

A thin epithelium covers the surface of the yolk mass and is continuous with the walls of the pharynx. The epithelium of the upper surface from the tip of the endostyle to the oesophageal opening is called the retro-pharyngeal raphe (Brien, 1929) (Figs. 2A, 3G). With each contraction the raphe is shortened and the yolk organ is gradually eliminated from its larval association with the pharynx to a position posterior to it, the two assuming a linear relationship with no communication between them. Once the separation is effected, the structure can be called the epicardium. It continues to lie parallel with the digestive loop. The yolk organ in Amaroecium has, therefore, the same significance it has in the other Aplousobranchiata, so far described, where it is similarly transformed directly into the epicardial tube.

The heart and its surrounding pericardial sac, being located at the distal end of the U-shaped intestine and epicardium or yolk organ, are involved in the rotating process to the same extent as the adjoining organs. The motion of the heart is passive insofar as it remains unchanged in structure during this time but within the first hour it commences to beat rhythmically.

Three hours after the expulsion of jelly from the papillae all rearrangement of larval systems into adult organization has advanced to the state where the animal is easily recognized as a tunicate. Within five hours the body regions characteristic of tunicates are clearly marked off: the thorax with its prominent endostyle and four horizontal rows of clefts; the abdomen lodging the epicardium and U-shaped digestive tract; the post-abdomen, in rudiment, but containing the crescent-shaped pericardium curved around the distal end of the blunt epicardium. The larval sensory vesicle is still evident. The tail structures are unchanged and protrude from the wall of the abdomen (Figs. 3, 4).

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[1] Compactness of body and pressure of atrium into pharynx are the explanation for two previous errors in reporting the number of gill clefts as 3 instead of 4 (Scott, 1946).

FIGURE 4. Continuation of the series showing assumption of adult body form and differentiation of adult organization. A, 5 hours; B, 12 hours; C, 18 hours; D, 24 hours; E, second day; F, third day after fixation. A, B, C, 150 ×; D, 130 ×; E, F, 60 ×. Animal has begun to take food in E, second day. Abd., abdomen; an., anus; d.l., dorsal languet; end., endostyle; ep.c., epicardial cavity; ep., epicardial tube; gl.st., glandular stomach; ht., heart; lan., languet; m.int., mid-intestine; oes., oesophagus; p.abd., post-abdomen; per., pericardium; p.g., peripharyngeal groove; ph.ph., prebranchial pharynx; p.st., post-stomach; th., thorax; y.m., yolk mass.

Throughout the period of rotation and beginning about fifteen minutes after fixation, mesenchyme cells of the body cavity migrate through the body wall and pass into the tunic. They are stellate in shape and produce long fibrils which extend from one cell to another. They move slowly towards the periphery of the tunic which continues to form in advance of them. The primary test of the tadpole is a secretion of the mantle. Cells continue to migrate through the body wall and they spread through the tunic.

Part of the process of reorganization includes the adaptation for adequate feeding and, therefore, freedom of movement of the oral end of the ascidiozooid. This is accomplished by flexion of the pharyngeal region on the rest of the body into an upright position so that the siphons are erected into the water. It occurs early in metamorphosis, within ten minutes, and is an action independent of the rotation movements. At the end of metamorphosis the abdomen and post-abdomen lie horizontally on the substratum to which they are attached while the thorax projects freely into the water at a right angle to the posterior body.

Further differentiation of the adult systems

When the ascidiozooid has rotated into its final axis the organs are disposed in their adult organization. The subsequent process of metamorphic differentiation is concerned with the elaboration of the systems to their mature state.

The pharynx extends into prominent occupation of the thorax. Dorsal languets project into the cavity to the left side of the points of intersection of the three horizontal branchial sinuses with the dorsal longitudinal one (Figs. 2B, 5E, F). They are similar in structure and in location in all Aplousobranchiata. The endostyle is differentiated into glandular and ciliated regions, consisting of five sections on each side. The center of the furrow is occupied by the flagellated band. On each side are the two basal glandular masses of cells separated from each other by a deep but narrow inferior ciliated band. Above the crescent shaped masses is the very slender "superior ciliated band" connecting the third glandular area with the rest and permitting it to spread widely into the floor of the pharynx (Fig. 6H). Branchial clefts number seven in each half row, tapering in size from smaller ones at both ends to larger ones in the middle.

The buccal siphon projects prominently as a rounded eminence, its collar cleft into six blunt lobes, its aperture deeply indented to the velar base which is divided into tentacles. Below the velum is the pre-branchial chamber limited by the peripharyngeal grooves into which the ciliated funnel of the hypophyseal duct enters. The peripharyngeal grooves connect the endostyle with the dorsal lamina (Figs. 2C, 4D).

The atrial siphon is less conspicuously elevated from the surface and its shallow rim remains undivided. The aperture communicates with the common or cloacal chamber of the peripharyngeal cavities. Both apertures have a layer of tunic lining

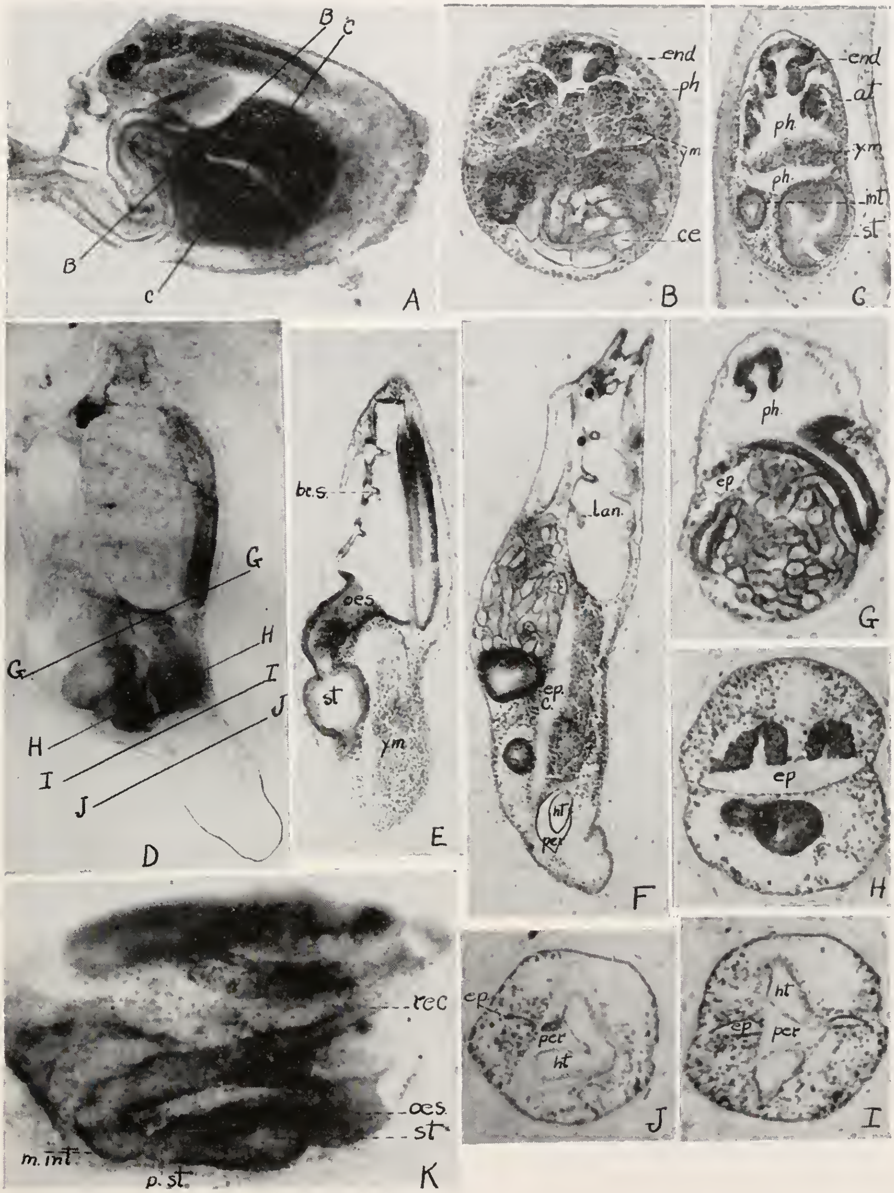
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FIGURE 5. A, Whole mount of tadpole at moment of fixation with lines to locate transverse sections in B and C. D, Whole mount of ascidiozooid 12 hours after fixation showing locations of transverse sections in G, H, I and J; E and F, longitudinal sections through D; K, Whole mount of tadpole at beginning of metamorphosis showing early constriction of digestive tract. A, 200 ×; D, 150 ×; K, 250 ×. at., atrium; br.s., bronchial sinus; c.e., caudal elements; end., endostyle; ep.c., epicardial cavity; ht., heart; int., intestine; lan., languet; m.int., mid-intestine; oes., oesophagus; per., pericardium; ph., pharynx; p.st., post-stomach; rec., rectum; st., stomach; y.m., yolk mass.

the collar but until the end of the first day of metamorphosis they are both closed with a plug of tunicin.

Between the two siphons lies the neural equipment, otolith and eye in a sensory vesicle to the right, neural ganglion and subneural gland to the immediate left and visceral or larval ganglion below them connecting with the neural tube of the tail mass (Fig. 3). The latter, still unchanged, presents a prominent bulge on that side of the body.

The epicardium and digestive loop lie posterior to the pharynx in the abdomen which is not, at this time, sharply demarcated from the thorax. Both structures end at the same level. Pericardium and pulsating heart form a clear crescent immediately behind them, the area of the body cavity enclosing them being the only indication of post-abdomen present toward the middle of the first day. The three body regions of tunicates are, therefore, evident but not completely developed (Fig. 4).

The remaining period of metamorphosis may be considered under the following activities:

(1) Body growth

In the course of two days the body completes its development into the adult form. The thorax expands into a transparent urn opening through a deep collared siphon crowned with six tapering lobes. The line of the buccal orifice curves through 90° to the atrial siphon communicating with the correspondingly expanded atrial cloaca. The abdomen assumes definite regional characteristics with elongation of the intestinal loop to its full length and reduction in width of the proximal epicardium. Early in the second day, 30 hours after fixation, presence of food and associated contents in typical pellet shape makes the abdomen opaque. Continued extension of the body in the long axis produces the attenuated post-abdomen. The major length of the epicardium with its light orange deutoplasm, the pericardium and closely packed cells of the body cavity impart a faintly mottled appearance to this region. A circular rim of tissue at the level of the pericardium and distal end of epicardium holds the ends of the muscle fibres of the mantle beyond which the post-abdominal tip is completely transparent (Figs. 2 and 4).

The processes of expansion and extension are attended by mitotic activity, especially in the more compact tissues like endostyle, digestive tract, and mesenchymatous cells. Epidermal cells show scattered divisions also but the fact that the tissue is spread into a thin layer makes the figures less evident.

(2) Differentiation of the digestive tract

The digestive tract constricts into regions. The oesophagus is a broad tube curving from a funnel-shaped pharyngeal orifice to the cardiac constriction. The stomach is a short, bulbous distension, abruptly narrowed posteriorly where it continues into the post-stomach or pyloric stomach (Figs. 2, 4, 5K). During the second day of development, growth in diameter of the stomach results in the formation of about 20 longitudinal crypts characteristic of the stomach of *Amaroecium constellatum* (Figs. 4F, 6D). Histological differentiation occurs and secretion is in progress when feeding begins.

The valve at the pyloric end of the stomach opens into the short, vertical limb of mid-intestine extending to the curve of the loop where a second constriction separates it from the final division of the intestine or rectum which ascends from that point to the floor of the atrium. When feeding commences, the mid-intestine compresses the mucus-bound contents into ovoid pellets and they retain that shape until their expulsion at the anus (Figs. 4F, 6A).

Histological differentiation is confined to the organization of epithelium typical

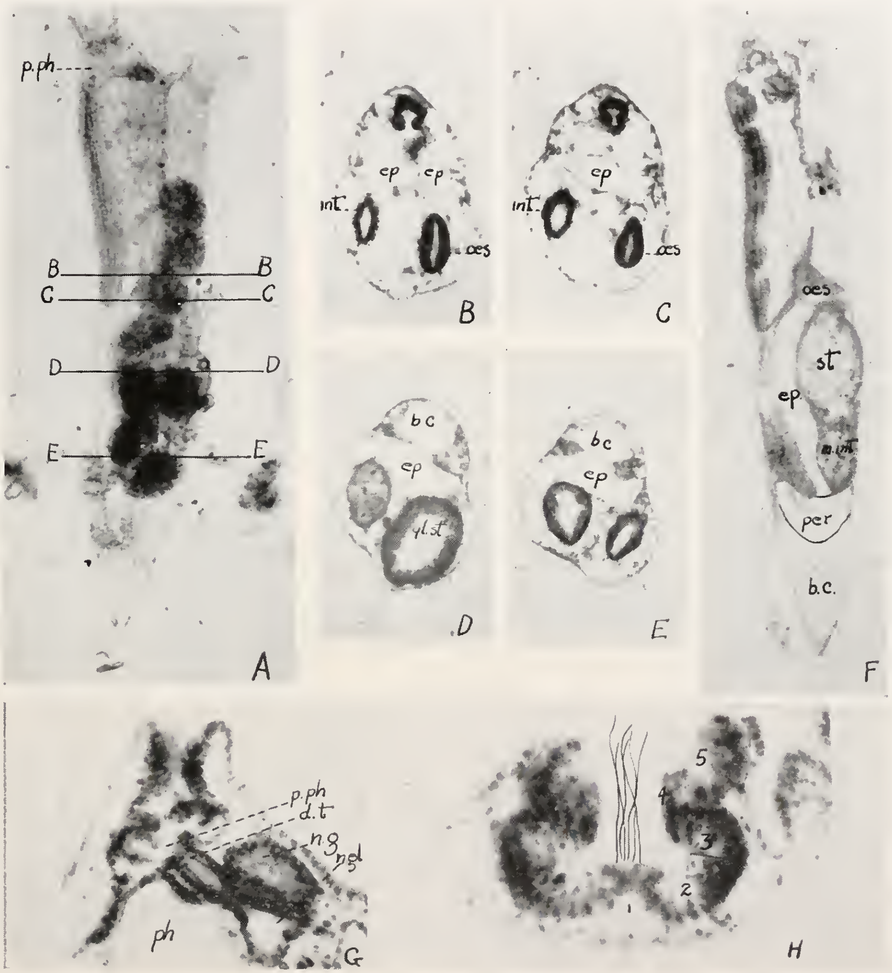

FIGURE 6. A, Whole mount of 2-day old ascidiozooid marked to locate transverse sections in B, C, D and E. F, longitudinal section through A. G, section through nervous system of adult. H, detail of endostyle with regions indicated. A, F, 100 ×; B, C, D, E, 150 ×; G, 450 ×; H, 1000 ×. B.c., body cavity; d.t., dorsal tubercle; ep., epicardium; gl.st., glandular stomach; int., intestine; m.int., mid-intestine; n.gl., neural gland; n.g., neural ganglion; oes., oesophagus; per., pericardium; ph., pharynx; p.ph., prebranchial pharynx; st., stomach.

of the various segments of the digestive tract; deep, slender columnar cells in oesophagus and mid-intestine; longitudinal glandular folds in the stomach; shallow columnar cells in the rectum. At a later stage, after budding has progressed, the pyloric gland of ascidians develops from the surface of the rectum to the pyloric stomach.

(3) The epicardium

When it is excluded from the pharyngeal cavity by shortening of the retropharyngeal raphe the epicardium is a broad, flattened cavity, the floor of which is composed of the nutritive endoderm flexed into two narrow clefts connected by thin epithelium (Fig. 5H). The roof is a continuation of the thin squamous epithelium that provides the yolk organ with its covering during the earlier stages of development. The cavity is identical with the one that becomes evident between the digestive loop and the deutoplasm during the period of rotation (Figs. 3, 4). Differentiation of the epicardium consists of elongation of the two yolk-lined furrows into long slender grooves extending through the abdomen and lengthening post-abdomen (Fig. 5E, F). At anterior and posterior ends the furrows retain their individuality but lack the yolk content and the epicardium is, therefore, double at both ends (Fig. 6A–F). By the end of the second day much of the yolk is consumed but the two narrow strips of light orange deutoplasm remain to identify the epicardium throughout the length of the post-abdomen a little beyond the pericardium. The quantity of yolk is greater at the tip and decreases in amount progressively to total absence toward the pharynx (Fig. 4).

(4) Pericardium and heart

Pericardium and enclosed heart change little except in size and position throughout the period of metamorphosis. The heart is tightly constricted in its middle and opens at each end, where it remains attached to the pericardium by a narrow neck into the sub-endostylar and perivisceral sinuses respectively (Fig. 2C). The pericardium is spacious in size and curved into a crescent with distal tips of the epicardium lying at right angles to it (Fig. 5 I, J). The pericardial sac maintains its location at the distal end of the elongating post-abdomen where it pulsates conspicuously.

(5) Degenerative changes

Those organs belonging exclusively to the larval action system or "transient organization" (Berrill) are gradually eliminated by various disintegrating processes.

The sensory structures and associated ganglia begin to disintegrate about five hours after fixation, fragments of the pigment being found in any part of the body from that time until the end of metamorphosis (Figs. 4, 5). The neural ganglion, previously called the definitive ganglion, and the hypophyseal gland with its ciliated duct remain as the adult neural elements. The gland at metamorphosis is a vesicular enlargement under the ganglion, and at its posterior end. It opens into the basal chamber of the buccal siphon by a ciliated duct with a funnel-shaped aperture called the dorsal tubercle. The region of the oral siphon is referred to as the prebranchial pharynx by Berrill. It lies between the peripharyngeal bands and the velar tentacles (Fig. 6G).

The caudal mass begins to break up during the latter part of the second day. The chordal, muscle, and neural cells lose their identity and clumps of them are scattered about through the body cavity. After their disruption the body is uniformly slender except for the expanded thorax holding itself erect on the recumbent abdomen and post-abdomen (Fig. 4).

Discussion

Descriptions of metamorphosis in ascidians are confined, in general, to considerations of the morphological aspects of the reorganization of larval systems into the adult. Explanations have been proposed to account for rotation on the transverse axis in various degrees of angles from 90° to 180°. Seeliger (1885) describes in Clavelina a pocket of epidermis depressed between the papillae and the oral siphon which is everted to the surface to assist in carrying the siphons through the arc to their position opposite the point of attachment. The pocket is "spare" tissue provided during embryonic development for the lengthening of the body on that side.

Observation of metamorphosis in Amaroecium in the living state is a convincing demonstration of the active role played by contractions of the body in reorienting the animal on its axis. The muscle strands developed in the mantle of the larva effect the movements.

The larva ingests neither food nor water and, therefore, the contractions can alter the shape of the flexible organs without impairing respiratory or digestive activities. All parts of the digestive tract are involved to the extent that the enteron is literally pushed into a tortuous mass toward the oesophageal funnel. The oesophagus and oral siphon retain their relationship; the cloacal chamber of the atrium and the anus retain their relationship; the convoluted loop of stomach and intestine, at the end of the contraction period, again extends itself in length but into a straight angle with the siphon, having rotated thus through an angle of 90°.

The endostyle is a densely cellulated structure in the tadpole and plays no active role in the contractions. It may buckle into one or two sharp folds and it always becomes arched during the height of the torsion but it retains its rod-like appearance. It is moved in hinge-fashion through an arc of 90° with the siphonal region. The yolk organ is inflexible but dilation of the pharynx and extension of its wall to the length of the endostyle in the new axis presses it out of the pharyngeal cavity into the space cleared by the telescoping effect of contraction on the enteric loop. Brien (1930) refers to the exclusion of the deutoplasmic mass from the pharynx as a shortening of the retro-pharyngeal raphe in Fragarium. He does not refer to the part played by the contractions of the mantle as initiating the process but the change in relative length of the epithelium between oesophagus and tip of endostyle is the same in both ascidiozooids.

The differentiation or dedifferentiation of the epicardium from the yolk mass is similar, also, in both animals, Fragarium and Amaroecium, being a direct conversion of the nutritive yolk into the slender, yolk-retaining epicardial tube of the abdomen and post-abdomen. It may be a characteristic phase of metamorphosis in the heavy-yolked Aplousobranchiata. In general, the epicardium of ascidians is produced by evaginations of the pharyngeal floor.

During the progress of metamorphosis it is possible to see the migration of mesenchyme cells through the mantle into the tunic. They move slowly through the tunicin until they establish a loose network with their long processes. The cells constantly change shape and the tunic becomes thicker but it is impossible to ascertain whether the cells are adding part of the secretion from their activity. Berrill (1950) is of the opinion that all tunic is secreted by the mantle or epidermis. An analysis of some experimental work which will follow in a subsequent paper leaves no doubt that the mantle is the seat of secretory activity early in the period of metamorphosis. This is true particularly in the area of attachment where the secretion effects adhesion to the substrate. The adhesive papillae provide for the preliminary attachment which is of transitory character, the test vesicles provide some of the cementing tunicin which is effective during the early stages of metamorphosis. Their secretory function is of short duration and is restricted to the region distal to the siphons. It seems that the test vesicles provide attachment for the contraction period when anchorage is required for the strong body movements of rotation and erection and before the mantle commences its secreting activity. The fact that the mesenchyme cells are in full march through the mantle when the tunic thickens uniformly over the surface of the ascidiozooid indicates that the final tunic may be the result of the combined activity of mantle and mesenchyme cells of the body cavity.

The points of discussion are of no controversial value but they clarify certain phases of metamorphosis in tunicates that have not been considered previously and, also, they leave another point, the origin of the tunic, to further study and analysis.

Summary

1. Metamorphosis in Amaroecium commences with the explosive release of secretion by the adhesive papillae and the steady, but rapid withdrawal of the caudal contents into a compact identifiable mass at the posterior end of the tadpole's trunk.

2. Reorientation of axes is initiated by strong contractions of the larval body which mark the beginning of reorganization in the internal organs.

3. The digestive loop, endostyle, yolk mass, and heart rotate through an arc of about 90° to their adult alignment by the action of the contractile force.

4. The pharynx expands and increases in depth being accompanied in its extension by the atrial or peripharyngeal cavities, the four rows of gill slits coming prominently into view.

5. Epicardial tube is differentiated directly from the yolk mass.

6. Typical thoracic, abdominal and post-abdominal regions of the tunicate body are developed by the end of the first twelve hours.

7. Ganglion and subneural glands are transformed into the adult nervous system while the products of disintegrating sensory pigment are scattered through the body.

8. Cytolysis of the caudal tissues begins after the adult organization has completely replaced the larval organization.

9. Reorientation of axes is accomplished within an hour after fixation of the larva; metamorphosis may be considered as completed within forty-eight hours.

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THE CONTROL OF CEMENT GLAND DEVELOPMENT IN THE CRAYFISH, CAMBARUS[1]

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The previous work concerned with elucidating possible hormonal mechanisms in the control of secondary sexual characteristics in the Crustacea has been recently reviewed by Brown (1952). That such work has to date been unsuccessful in providing indisputable evidence for an influence of endocrine secretions on these characteristics may be explained in part by reference to technical obstacles in the path of a direct application of standard endocrinological techniques to decapods. The position of the gonads in this group, together with problems imposed by the brittle, calcified exoskeleton, have rendered gonadectomy so difficult that it has not yet been successfully attempted. Takewaki and Nakamura (1944) were able to castrate an isopod, *Armadillidium,* but found no apparent influence on the development of the oöstegites in females.

The second basic problem encountered in attempting to study hormonal influences on the development of secondary sexual characteristics lies in the nature of the characters which have been considered. Those secondary characters which appear cyclically in relation to the breeding season would appear, *a priori,* to be those most readily influenced by fluctuations in hormonal balance. However, those which have been studied in this connection to date have the disadvantage of appearing as discontinuous modifications in form at the time of molt. Thus, their occurrence tends to be of an all-or-none character which renders studies of developmental rates difficult.

The cement glands of the Astacura may be considered secondary sexual characteristics on the basis of their sexually dimorphic character and the intimate relation between their discharge and the process of egg-laying. They are usually assigned the function of producing the cement which attaches the eggs to the pleopod setae of the female. Reviews of earlier literature concerning the function of these glands are provided by Broekhuysen (1936) and Yonge (1937).

Andrews (1904) described the process of egg-laying and glaire secretion in *Cambarus affinis* and stated that the cement glands in this form are chiefly found ventrally beneath the abdominal sterna and in the abdominal appendages. Farkas (1927) conducted a detailed histological study of the glands in *Astacus* and stated that the cement glands are essentially similar to the other tegumental glands described in his work. Two types of cells can be distinguished in the glands: relatively large duct cells and smaller secretory cells. The duct cells produce an intracellular system of ducts which supplies the secretory cells and provides a pathway for the discharge of their mucin-like secretion.

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These glands are particularly suitable for studies of possible hormonal influences on their development since their maturation occurs as a continuous process without the necessary intervention of molt. Yonge (1937) suggested the possibility of humoral control of their development but did not attempt to support this possibility. Lloyd and Yonge (1940) considered a correlation noted between the mature condition of the ovary and the cement glands in *Crangon* to be evidence for the existence of a hormone elaborated by the ovary and responsible for cement gland development. No attempt has been encountered to study possible hormonal influences on the cement glands using classical endocrinological techniques.

An important reproductive function of the crustacean sinus gland was first demonstrated for the shrimp, *Leander*, by Panouse (1943, 1944, 1946) and subsequently confirmed for *Cambarus* and *Uca* by Brown and Jones (1947, 1948). In these forms, sinus gland removal results in an increased rate of ovarian maturation. The work of Stephens (1952) provides evidence for a role of materials elaborated by the cerebral ganglia and circumesophageal connectives in the sexual cycle of *Cambarus*. Consideration of these results directed attention in the following work to the sinus gland and the central nervous system as possible sources of tropins concerned with cement gland development.

Material and Methods

The experiments to be described were performed with groups of *Cambarus rusticus* obtained from Ohio and with groups of *Cambarus virilis* collected in the vicinity of Chicago, Illinois. Reference is also made to scattered observations of other species of *Cambarus* collected locally. The protocol of each of the several experiments performed will be described subsequently.

The operative procedures were: (1) removal of both eyestalks, and (2) implantation of central nervous organs in the ventral abdominal haemocoel. The eyestalks were removed at their bases with a scalpel, thus removing the sinus glands and associated structures. The eye stubs were cauterized immediately to avoid excessive blood loss. In the case of animals receiving implants of central nervous tissues, the cerebral ganglia and circumesophageal connectives were removed from donor animals and implanted at once in a medium of van Harreveld's solution with a tuberculin syringe bearing a 16-gauge hypodermic needle. Animals were not fed in the course of any experiments.

The distribution of the cement glands will be described for female *Cambarus rusticus*. No differences in distribution were observed in other species of *Cambarus* employed. The glands underlie the second through the sixth abdominal sterna and extend laterally into the anterior region of the associated pleura. They also occur in the protopodites, endopodites and exopodites of the last five pairs of abdominal appendages. The pores through which the cement glands discharge their secretion open independently of the setae of these appendages.

In order to assess the results of experimental treatment on the development of the cement glands, a series of arbitrary stages based on their gross appearance at various points in their development has been used. Although the gross appearance as described for each stage is much the same in all of the cement glands in a given animal, it is convenient to confine attention to the ventral surface of the uropods. The stages have been defined as follows:

Stage 1: The distinguishing characteristic of this stage is the appearance of milky-white, translucent, circular or subcircular areas, approximately 0.1 to 0.2 mm. in diameter, marking the position of the future cement gland pores. There may be 30 to 45 such areas on each ramus of a uropod depending to some extent on the size of the animal. An examination of sectioned material indicates that these areas are correlated with the appearance of the distal duct cells of the cement glands. A considerable number of ducts is associated with each presumptive pore, each duct being only about five micra in diameter.

Stage 2: The white areas described above persist and subcircular, transparent areas appear within their confines ranging from 0.05 to 0.1 mm. in diameter (Fig. 1). These transparent areas are the cement gland pores which penetrate through the chitinous layers of the integument to the non-chitinous epicuticle (Fig. 4).

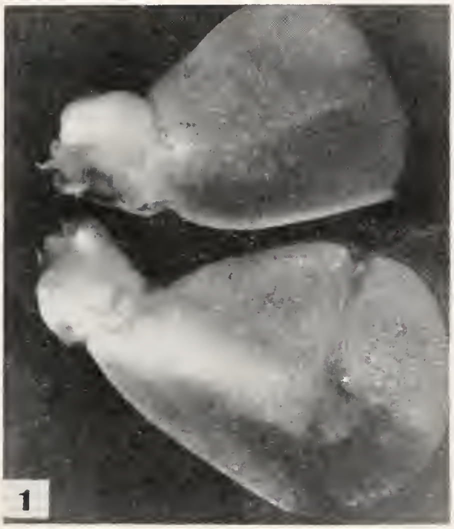
Stage 3: At this stage the secretory cells have appeared in close association with the inner duct cells and are just beginning secretory activity. The glands can be seen as translucent, white, lobate clusters surrounding the cement gland pores in an irregular fashion. Each lobe represents a discrete gland, many glands providing independent ducts to one pore. Figure 3 is a photograph of a histological section of cement glands at this stage.

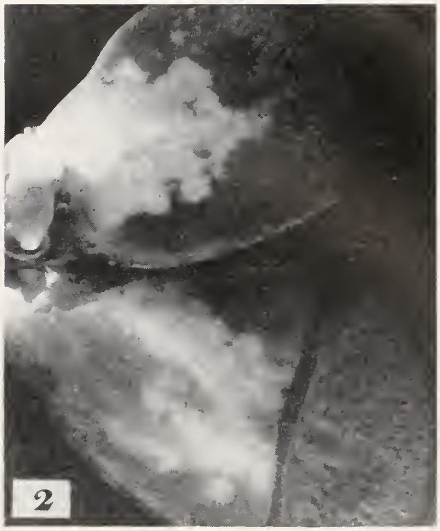
Stage 4: The previously described elements are still clearly visible and in addition the cement glands have become striking, opaque, cream-white as a result of engorgement with dense mucoid secretion. Figure 2 illustrates the gross appearance of cement glands which are approaching this stage. However, the cement glands eventually become so heavily charged with secretion and so distended that it is difficult to make out their lobate character. Stage 4 is the terminal or mature stage of the cement glands.

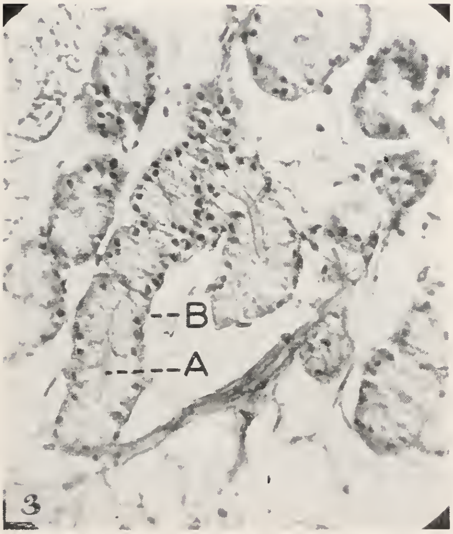
Intermediate stages of cement gland development can be defined quite accurately. Thus an animal with patent stage 1 areas plus about half the cement gland pores would be classified as stage 1.5. Such interpolations allow staging to quarter units throughout the scale. The accuracy of such determinations has been ascertained by re-reading groups of animals within an interval so brief that no significant change could have occurred. Using a group of 10 animals, the average reading checks within 0.05 unit.

One source of error was encountered in using this system. When experimental conditions are such that no cement gland development is occurring, the translucent areas surrounding or portending the pores may fade. Since they normally limn the pores, superficial examination may fail to reveal all the pores present which would produce a low reading. It was also ascertained that these areas disappear just following molt but normally reappear within 48 hours after molt.

The fading which is observed in animals which are not in immediate post-molt stages can be considered retrogressive development or it can be interpreted as a concomitant of developmental arrest. The latter alternative has been chosen in the analysis of data in this paper. Thus decrements measured in comparing stages of the same animals taken at different times are reported but are simply treated as zero increments in the calculation of averages. The alternative procedure would







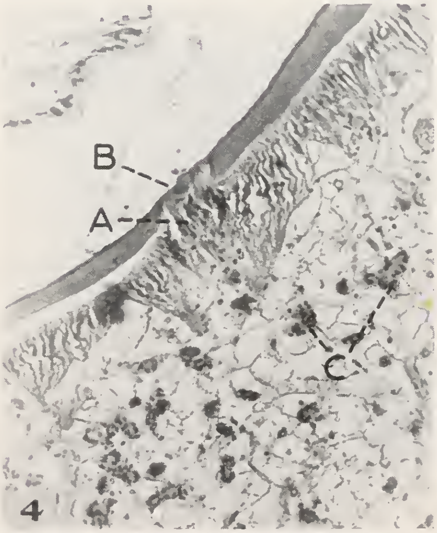


FIGURE 1. Gross appearance of the uropod of a female *Cambarus rusticus* with the cement glands at stage 2.0 (× 8).

FIGURE 2. Gross appearance of the uropod of a female *Cambarus rusticus* with the cement glands at stage 3.5 (× 8).

FIGURE 3. Histological section through uropod showing cement glands at stage 3.0. The ducts (A) can be seen lying within the masses of gland cells (B) (× 220).

FIGURE 4. Histological section through uropod showing the cement gland ducts (A) and a cement gland pore (B). Gland cells (C) which have appeared in association with the inner duct cells but have not yet begun secretion may be seen in this section (× 120).

not materially influence the conclusions suggested by the data. Animals which had molted less than two days previous to the time of a reading were not considered. Finally, it should be stressed that development as evaluated by means of the scale described above is not known to proceed at a uniform rate.

Experiments and Results

A. *Influence of destalking and central nervous system implants*

In the course of these experiments, it became apparent that the mature *Cambarus rusticus* females employed were responding to experimental treatment in a quite different fashion from the small *Cambarus virilis* females. It will be argued later that these *C. virilis* were immature. In any event, it is simplest to discuss the experiments employing these different species separately.

1. *Cambarus rusticus*

A preliminary experiment was performed over the period from July 4 to July 13, 1951. Three groups of females ranging in carapace length from 2.5 to 3.9 cm. were employed.

Group A consisted of 40 animals of average carapace length 3.26 cm., destalked on July 4. Group B consisted of 40 animals of average carapace length 3.26 cm., similarly destalked on this date and receiving one implant of nervous tissue from female *C. rusticus* donors on July 8. Group C was comprised of 15 untreated animals whose average carapace length was 3.13 cm. and served as a control group. All animals were maintained in separate compartments in running water at an average temperature of 22.6° C.

The staging system for evaluating the condition of the cement glands was not yet developed at the time these animals were isolated and consequently no values are available for the initial condition of their cement glands. Readings of each surviving animal in each group were taken on July 13, nine days after destalking and five days after implanting the groups so treated. These observations are arranged in tabular form in Table I.

A trend toward more advanced cement gland development with increasing size is apparent in all groups. However, there is considerable variation within a given size range. In an attempt to compensate for this variation, the average cement gland stage has been calculated for a series of overlapping size ranges and is presented graphically in Figure 5. This graph shows that the average development of the cement glands in those animals which were destalked and received nervous tissue implants is quite consistently more advanced than the condition manifested by the controls and those animals which were destalked but received no implants.

An additional control group was selected from the stock animals on July 13 and the cement glands staged. Although the variation between control groups was considerable, both control groups were clearly less advanced in cement gland development than the implanted animals. These results are suggestive of a possible tropic influence of the nervous system on cement gland development. In order to test this possibility further, the following experiments were performed.

Experiment 1. Two groups of female *Cambarus rusticus* ranging in carapace length from 1.7 to 2.8 cm. were considered in this experiment. Group A consisted of 14 animals, destalked on August 22, whose average carapace length was 1.92 cm. with an average cement gland stage of 0.77. Group B consisted of 14 animals which were destalked on August 22 and received one implant of nervous tissue

TABLE I

Initial experiment with Cambarus rusticus. Condition of the cement glands of each animal in each group on July 13, 1951

Carapace length (cm.)	Destalked	Cement gland stage destalked, implanted	Control
2.5	1.5		
2.6			1.0
2.7		1.5	2.75
2.8	0.5		1.5
	1.0		1.0
2.9	1.0	1.5	1.75
	3.0	2.0	1.5
	1.25		
3.0		2.25	3.0
		1.25	
		3.0	
		3.0	
3.1	1.25	2.0	1.25
	1.5	2.0	
		1.5	
3.2	2.0	3.0	3.0
		2.0	
		1.5	
3.3	3.25	3.0	3.0
	3.0		
	3.5		
	1.5		
	1.5		
3.4	3.0	3.25	
	2.0	3.5	
	3.0		
3.5	2.0	3.5	2.0
3.6	1.5	3.0	2.75
	3.0		2.25
	3.0		
	3.5		
3.7	2.0		
3.8	3.25	1.75	3.0

from female donors on August 26. The average carapace length of the animals in this group was 2.19 cm. and their average cement gland stage was 1.14. These animals were maintained in separate compartments in running water at an average temperature of 22.5° C. The state of the cement glands was assessed on August 26 and again on August 31. The increments for the animals in each group are tabulated in Table II. The average increment for each group was:

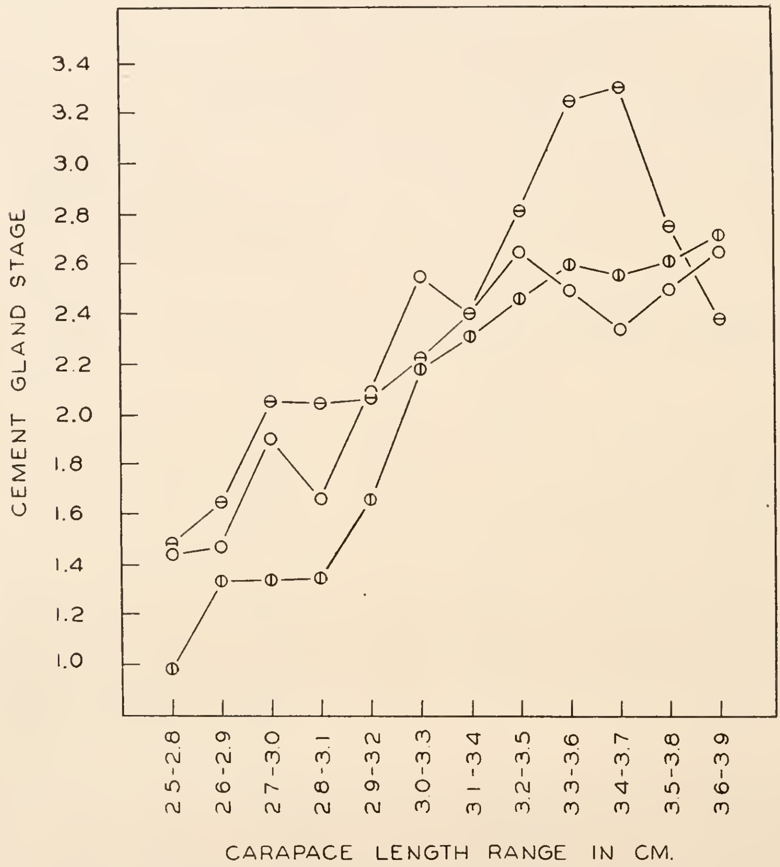


FIGURE 5. Graph showing the average cement gland stage on July 13, 1951 for the size ranges indicated of animals in groups A, B, and C of the initial experiment with *Cambarus rusticus*. Group A (destalked) ⦶. Group B (destalked, implanted) ⊖. Group C (untreated controls) ○.

Group A (destalked, 9 surviving)	0.53
Group B (destalked and implanted, 9 surviving)	0.45

It should be pointed out at this time that group A in this experiment is not comparable to any other group considered because of its low initial cement gland condition.

TABLE II

Increment in cement gland stage measured over the period indicated in experiments with C. rusticus and C. virilis

Cambarus rusticus

Experiment 1		Experiment 2		
Group A (destalked)	Group B (implanted)	Group A (destalked)	Group B (implanted)	Controls (9/6 to 9/14)
1.25	0.25	0.25	1.25	0.0
0.25	1.25	0.0	1.0	0.25
1.0	0.25	0.5	0.25	0.0
0.25	0.25	0.75	0.0	0.0
0.5	0.0	0.75	1.0	0.0
0.75	0.75	0.0	1.5	0.0
0.0	1.0	0.25	1.25	0.25
0.75	0.25	0.5	1.0	−0.25
0.0	0.0	0.25	0.75	−0.25
		0.0	0.0	−0.25
				−0.25
Av. = 0.53±0.15 $\sigma=0.44$	Av. = 0.45±0.15 $\sigma=0.445$	Av. = 0.325±0.092 $\sigma=0.29$	Av. = 0.80±0.17 $\sigma=0.54$	Av. = 0.05±0.032 $\sigma=0.101$

Cambarus virilis

Experiment 1		
Group A (destalked)	Group B (implanted)	Group C (control)
0.0	0.0	0.0
0.0	0.0	0.0
−0.25	−0.25	0.0
−0.25	−0.25	0.0
−0.25	0.25	0.0
		0.0
Av. = 0.0±.0	Av. = 0.05±0.06	0.0
		0.25
$\sigma=.0$	$\sigma=0.12$	0.25
		0.25
		0.25
		0.25
		0.25
		0.25
		Av. = 0.125±0.036 $\sigma=0.13$

Experiment 2. Two groups of *Cambarus rusticus* ranging in carapace length from 1.7 to 3.2 cm. were considered in this experiment. Group A consisted of 11 animals destalked on September 20. The average carapace length of animals in this group was 1.89 cm. and their average cement gland stage was 1.66. Group B consisted of 20 females which were destalked on September 20 and received one implant of nervous tissue from female donors on September 22. The average

carapace length of this group was 2.10 cm. and their average cement gland stage was 1.63.

These animals were maintained in separate compartments in running water at an average temperature of 19.6° C. The condition of the cement glands was assessed on September 22 and again on September 28. The increments for the surviving animals in each group are tabulated in Table II. The average increment for each group was:

Group A (destalked, 10 surviving)	0.325
Group B (destalked and implanted, 10 surviving)	0.80

A control group of 14 untreated animals was maintained over the period from September 6 to September 14 to provide information concerning the normal rate of cement gland development under the laboratory conditions described. These animals were maintained in running water in separate compartments at an average temperature of 21.2° C. The condition of the cement glands was assessed on the dates mentioned above and the increments for the surviving 11 animals are tabulated in Table II. The average increment for this group was 0.05.

Before concluding the presentation of the results of these experiments, one important fact should be mentioned. The increments reported in state of cement gland development in destalked animals and in animals receiving implants after destalking are not the initial portion of a continuing increase in developmental state but rather an induced spurt of development which does not persist until the glands attain maturity. Mortality in these experiments was high and consequently observations are scattered, but no instance has been observed where induced development involved a total increment of more than 1.5 stage units. In general, no increment, or only a very small one, is measured after an initial ten-day to two-week period of response. Animals have been maintained as long as 45 days after destalking and show no substantial advance over their cement gland condition 10 days after this operation.

2. *Cambarus virilis*

Experiment 1. Three groups of *Cambarus virilis* females collected from a small artificial lake in the vicinity of Chicago, Illinois were employed in this experiment. Their carapace lengths ranged from 2.0 to 3.0 cm.

Group A consisted of 20 animals which were destalked on July 29. The average carapace length of the animals in this group was 2.48 cm. and their average cement gland stage was 1.44.

Group B consisted of 20 animals which were destalked on July 29 and received one implant of nervous tissue from female *C. virilis* donors on July 31. The average carapace length of the animals in this group was 2.54 cm. and their average cement gland stage was 1.46.

Group C was comprised of 20 untreated animals whose average carapace length was 2.54 cm. and whose average cement gland stage was 1.44. This group served as normal control for groups A and B.

All animals were maintained in separate compartments in running water at an average temperature of 23.0° C. The developmental condition of the cement glands was assessed on July 29 and again on August 8, 10 days after eyestalk removal and 8 days after implanting the groups so treated. The increments for the animals in each group over this period are tabulated in Table II. The average increment for each group was:

Group A (destalked, 5 surviving)	0.00
Group B (destalked and implanted, 5 surviving)	0.05
Group C (normal controls, 14 surviving)	0.125

Experiment 2. This experiment was performed with a total of 90 locally collected female *C. virilis* ranging in carapace length from 1.9 to 2.8 cm. The animals uniformly showed cement glands at stage 2.0. They were divided into five groups as follows:

1. Destalked on November 1.
2. Destalked on November 1 and received one implant of nervous tissue from female donors on November 3.
3. Destalked on November 1 and received one implant of nervous tissue from male donors on November 3.
4. Destalked on November 1 and received an implant of muscle tissue on November 3.
5. Untreated controls.

All animals were maintained in separate compartments in running water at an average temperature of 17.0° C. The condition of the cement glands was assessed on November 1 and again on November 13. No fewer than 11 animals in each group survived this period. In no case did any animal manifest any advance in cement gland condition, all animals remaining at stage 2.0. Subsequent implantation of nervous tissue from mature *Cambarus propinquus* female donors was similarly without effect on the cement gland development of the host *C. virilis*.

The following observations seem to indicate that the animals employed in this experiment were immature. The oöcytes in these animals were small, pale, translucent white with an average maximum observed diameter (23 animals, carapace length 2.0 to 2.7 cm.) of 0.237 mm. This is to be contrasted with the presence of olive-green oöcytes of diameters ranging from 1.5 to 2.0 mm. in normal mature *C. virilis* at this time of year. In addition, the gonopods of the males of similar size and collected at the same time were uniformly of form II (the sexually inactive or juvenile form) in contrast to the form I (sexually active) gonopods manifested by mature males at this season.

Similarly the oöcytes of the *C. virilis* females employed in the first experiment with this species were small and the gonopods of comparable males were of form II. However, this is less definite evidence of their immaturity since mature females may normally have small, white oöcytes in July. However, mature males are usually in form I by the end of July so we may tentatively argue that these animals were immature. The fact that the cement glands of these animals were in stages less than 2.0 might be taken to indicate that they were hatched in the same spring. However, in the absence of observations of the cement glands of yearling, immature crayfishes, this does not necessarily follow.

B. Additional observations and experiments

Two experiments were conducted to determine if the length of the daily light period to which the animals were exposed influenced the rate of cement gland de-

Table III

Cement gland stages of animals subjected to 20-hour light and constant darkness, assessed on the dates indicated

Carapace length	Cement gland stage					
	20-hour light			Constant darkness		
	7/17	7/24	7/29	7/17	7/24	7/29
2.1	1.0	1.0	1.0	0.75	1.0	0.75
2.2	0.75	0.75	1.25	0.75	1.0	1.5
				1.5	1.25	
2.3	0.75	1.5	1.5			
	1.0					
2.4	1.0	1.5	1.5	1.0	1.0	1.25
	1.0	1.25	1.5	1.0	1.0	
	1.0	1.0	1.0		1.25	
2.5	1.0	1.25		1.0	1.5	1.0
				1.0	1.5	1.25
2.6	0.5	1.25	1.5	1.5	1.5	1.5
	1.5	1.25	1.5	1.0	1.25	1.5
	1.0	1.25	1.75	1.5	1.0	1.25
	1.5	1.5	1.75	1.0	1.25	1.5
				1.5		
2.7	1.0	1.0	1.25	1.5	1.5	2.0
	1.5	3.0	2.5	1.5	1.0	1.5
	1.5	1.0	2.0	1.5	1.75	1.5
	2.0	2.0	1.5	1.25	1.5	1.5
	1.25	1.25	1.75	1.25	1.25	1.75
	1.25	1.25				1.75
	1.0	2.0				
	1.25	1.5				
	1.5	1.25				
2.8	1.5	2.5	1.5	1.25	1.5	1.25
	1.5	1.75	1.5	1.0	3.0	1.5
	2.0	1.75	1.75	2.0	1.75	1.5
	1.25	1.75	1.5	1.5	2.0	1.0
			1.5	1.0	1.25	1.5
				1.5	1.75	3.0
2.9	1.5	1.5	1.75	1.5	1.5	1.5
	1.5	1.0	1.25	1.5	1.5	1.75
	1.0	2.0	2.5	2.0	2.0	2.0
			2.0			
Av.	1.23	1.49	1.60	1.30	1.45	1.52

velopment. Again there is a sharp dichotomy in the response of the mature *C. rusticus* and that of the immature *C. virilis.*

In the first experiment two groups of female *C. rusticus* were maintained in running water, one in constant darkness and the other in 20-hour daily illumination. Group A, in 20-hour light, consisted of 28 females of average carapace length 2.61 cm. and an initial average cement gland stage of 1.23. Light was provided by two 25-watt incandescent lamps suspended from a wooden frame approximately 10 inches above water level, producing an illumination of from 8 to 50 foot-candles at the surface of the water. The lights were controlled by an automatic time clock and the tank shielded with black cloth to prevent the access of undesired illumination. Group B consisted of 26 animals of average carapace length 2.63 cm. and average initial cement gland stage 1.30 and was maintained in constant darkness. The animals in these two groups were not isolated. The average water temperature over the experimental period for group A was 23.0° C. while that for group B was 22.3° C.

The cement glands of each animal in each group were assessed on July 17, July 24 and July 29. These observations are tabulated in Table III. The increments may be expressed as units per day since the two periods are not the same. The averages are:

Twenty-hour light	
July 17 to July 24	0.037
July 24 to July 29	0.017
Constant darkness	
July 17 to July 24	0.021
July 24 to July 29	0.010

The cement glands of the animals exposed to 20-hour light seem to have developed more rapidly than those of the animals maintained in darkness for both periods. In both cases, development for the first period was more rapid than for the second. Observations were discontinued on July 29 because of an increase of mortality. Since the animals were not isolated, readings of only a portion of the original sample would be of dubious value.

A second experiment of similar design was initiated on November 7 with small *C. virilis* (average carapace length 2.21 cm.) whose cement glands were uniformly at stage 2.0. It was found that alteration of daily light ration did not suffice to induce development beyond this point in any of the animals.

The extent of cement gland development was positively correlated with the size of the animals in all groups examined with the exception of groups where all of the animals were at maturity and the *Cambarus virilis* referred to above whose cement glands were uniformly at stage 2.0. Examination of groups of *C. virilis, C. propinquus, C. immunis, C. rusticus, C. diogenes* and *C. blandingii acutus* indicates that this positive correlation is widespread among crayfishes. The type of evidence obtained is illustrated by Figure 5 where cement gland stage is plotted against carapace length. *C. rusticus* provided the largest size range over which this effect could be observed. As early as July 13, large females (carapace length of 4.5 cm.) were found with typical mature cement glands while on the same date an animal of carapace length of 1.5 cm. might show no trace of these structures.

A correlation between ovarian development and cement gland development was observed in *C. rusticus*. Ovarian condition was estimated by measuring the largest observed oöcytes with an ocular micrometer and a binocular dissecting microscope and recording this size as well as the color of the oöcytes. Such measurements were made for one hundred and five *C. rusticus* females at all stages of cement gland development from stage 1.0 to stage 4.0. These measurements are grouped according to cement gland development stage and tabulated in Table IV. The average values for these groups indicate that ovarian and cement gland development proceed in a

Table IV

Maximum observed oöcyte diameter in millimeters listed according to cement gland stage of the animals

Stage 1.0 −1.75	Stage 2.0 −2.75	Stage 3.0 −3.75	Stage 4.0
0.5	0.6	0.65	1.0
0.5	0.65	0.8	1.0
0.9	0.9	0.8	1.2
0.45	1.2	1.1	0.9
0.5	0.7	0.7	1.0
0.5	0.85	0.8	
0.7	0.7	0.9	
0.45	0.8	0.9	
0.4	0.55	0.8	
0.45	0.6	0.7	
0.55	0.8	1.0	
0.6	0.9	1.0	
0.6	0.6	0.9	
0.5	0.4	0.9	
0.45	0.9	1.0	
0.5	1.1	0.8	
0.6	0.6	0.85	
0.5	0.6	0.8	
0.45	0.65	0.9	
0.4	0.9	1.0	
0.6	0.6	0.4	
0.6	0.6	1.3	
0.7	0.7	0.5	
0.7	0.8	0.9	
0.7	0.7	0.8	
0.6	0.8	0.8	
0.7	0.7	1.0	
0.7	0.8	1.1	
0.7	0.7	0.7	
0.9	0.8	1.0	
0.6	0.7	0.3	
0.45	0.75		
0.6	0.6		
0.5	0.65		
	0.4		
Av. 0.575±0.019 $\sigma=.114$	0.720±0.028 $\sigma=.165$	0.842±0.036 $\sigma=.202$	1.02±0.049 $\sigma=.11$

roughly parallel fashion, though there is a considerable range of variation within each group. Similar data for other species of *Cambarus* indicate that this correlation is probably general in occurrence.

Discussion

It may be of value to provide a brief outline of events in the reproductive cycle of female crayfishes to form a basis for interpreting the results presented above. Reproductive cycles in *Cambarus* in the North Temperate Zone may be divided into two types on the basis of the time of egg-laying. Eggs are always hatched in the spring but may be laid in the fall and carried over the winter (*Cambarus immunis,* Tack, 1941), or they may be laid in the spring (*Cambarus propinquus,* van Deventer, 1937). In either case, the females present in a population in mid-summer may be divided into three groups:

1. Those animals which were mature and bore a clutch in the previous breeding season.
2. Those animals which failed to attain a minimal size necessary for reproductive activity in their first year, yearling immature females.
3. Those animals which were hatched in the preceding spring. This group can be logically subdivided into animals which will and those which will not attain the critical breeding size.

The cement glands of mature females revert to stage 0 (no gross indication of their presence) after the process of egg-laying. It is not known whether they begin to re-form immediately or there is a time lag before re-initiation of development. The cement glands of the yearling immature females attained stage 2.0 by the time of entrance into hibernaculae the preceding year. It is not known whether they continue their development from this point at the onset of spring or revert to stage 0 to re-initiate development of the cement glands. The third group of animals hatched in the preceding spring attains a stage of 2.0 or 4.0 by the following fall depending on their rate of growth and their success or failure to attain a minimal size for reproductive activity.

This cycle suggests that the correlation between size of the animals and extent of cement gland development observed during this study is not necessarily the result of a differential rate of development alone but may possibly be explained in part in terms of the three groups of animals in a population, outlined above.

Rates of cement gland development observed in unoperated animals may be listed to provide a basis for discussing variations induced by experimental operative treatment. These rates are expressed as average increments in cement gland stage per day.

Control group, *C. rusticus*	0.012
Control group, *C. virilis*	0.005
Twenty-hour light, *C. rusticus*	0.037
	0.017
Constant darkness, *C. rusticus*	0.021
	0.010

These rates may be compared with the *per diem* rates for destalked *C. rusticus* females and females receiving central nervous implants subsequent to destalking.

Experiment 1:	destalked	0.106
	destalked and implanted	0.090
Experiment 2:	destalked	0.054
	destalked and implanted	0.133

It is apparent that all of these rates clearly exceed any of the rates of cement gland development listed for unoperated animals. Thus the conclusion that destalking induces an increased rate of development of the cement glands seems justified. That this is not the result of the loss of the eyes in their capacity as photoreceptors is shown by the rate manifested by the unoperated animals in constant darkness. We may then conclude that some factor originating in the eyestalk, probably in the sinus gland, is serving to inhibit cement gland development in these animals.

The case for a central nervous tropic influence on the development of the cement glands rests on the initial exploratory experiment performed with *Cambarus rusticus* and on the results of the second experiment providing the data listed above. It was pointed out in describing experiment 1 with this species that the group of animals which was destalked was not comparable to any of the other groups considered because of the low initial stage of the cement glands. That this may have influenced the results is indicated by the fact that those animals with cement glands initially at stages less than 1.0 exhibited greater increments in this experiment than those with more advanced glands. However, although a role of central nervous neurosecretory elements in the control of cement gland development may be suggested, further experimental verification is necessary.

In no case did destalked *Cambarus virilis* females, whether receiving implants or not, exhibit significant cement gland development. The single increment measured in the implanted group of the first experiment (Table II) is within the limit of error inherent in the technique of estimation. It follows that removal of the sinus glands in these animals produced a cessation of development or failed to initiate development as contrasted with the increment in rate produced with similar treatment of *Cambarus rusticus*. Either the differences between the responses of *Cambarus rusticus* and *Cambarus virilis* to operative treatment are the result of a species difference in the controlling mechanism of cement gland development, or they are related to the fact that all of the *C. rusticus* were mature and all of the *C. virilis* were immature. In view of the close systematic relationship of the two forms, the latter explanation seems more probable.

These facts taken together suggest a controlling mechanism for cement gland development which closely parallels that postulated by Stephens (1952) for the control of ovarian development in *Cambarus virilis*. Thus the sinus gland would serve in the role of inhibitor (as shown by the increment in developmental rate in destalked, mature animals) and also elaborate a substance necessary either directly or indirectly for the development of the cement glands (as shown by the cessation of development in destalked, immature animals). The central nervous system in mature animals would serve either to store the sinus gland tropin or to convert it to an active form and store it, the rate of its release being under the control of the sinus gland inhibitor. A system with these characteristics would explain all of the reported observations but requires further experimental support.

Callan (1940) and Knowles and Callan (1940) demonstrated the failure of secondary sexual characteristics to develop in females of *Leander* following parasitic or x-ray castration. They suggested the possibility of an ovarian hormone controlling these characters but did not rule out the possibility of a general somatic effect of such treatment. On the other hand, Takewaki and Nakamura (1944) demonstrated that surgical removal of the ovary in the isopod, *Armadillidium*, did not influence subsequent oöstegite development.

Although a correlation is reported here between the state of ovarian and cement gland development, discrepancies in this correlation actually provide evidence against the gonadal hormone theory. The presence of early stages of cement gland development concomitant with an advanced ovarian condition might be explained in terms of a failure of tissue competence by a proponent of the gonadal hormone theory. However, in Table IV, two cases are presented in the third column which show a clear discrepancy in the other direction. These animals had cement glands in stages 3.0 and 3.5 and had white oöcytes of maximum observed diameter of 0.4 and 0.3 mm., respectively. The occurrence of such animals with advanced cement glands and small, immature oöcytes would appear to argue strongly against a direct dependence of the cement glands on the ovary. It may also be pointed out that Brown and Jones (1947) report a linear increase in dry-weight of the ovaries of destalked *Cambarus immunis* over a 53-day period which contrasts sharply with the short burst of development of the cement glands followed by relative quiescence produced by similar treatment. Thus it can be tentatively concluded that the control of the cement glands, at least, is not mediated by an ovarian tropin.

SUMMARY

1. The morphology and sequence of events in the course of normal development of the cement glands of female crayfish of the genus *Cambarus* are described and a technique for the study of the developmental rate of these glands is outlined.

2. In mature female crayfish, removal of the eyestalks produces an acceleration in the rate of development of the cement glands. Experimental evidence suggests that a further increment in rate may result when cerebral ganglia and circumesophageal connectives from mature females of the same species are implanted in the ventral haemocoel of destalked, mature animals.

3. Both destalking, and destalking with subsequent implanting of nervous tissue from immature female donors, lead to a cessation of cement gland development or fail to initiate development in immature animals.

4. The cement glands of mature females subjected to 20 hours of illumination per day appear to develop more rapidly than those of comparable animals maintained in constant darkness. However, modification of day-length is not sufficient to initiate cement gland development in quiescent immature females.

5. A correlation between developmental state of the cement glands and degree of oöcyte development is presented.

6. Characteristics of a possible humoral control of the cement glands are discussed as well as the contribution of this information to the more general subject of humoral control of secondary sexual characteristics in the decapods.

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AN ANALYSIS OF THE AGGREGATION STAGE IN THE DEVELOPMENT OF THE SLIME MOLDS, DICTYOSTELIACEAE. I. THE POPULATIONAL DISTRIBUTION OF THE CAPACITY TO INITIATE AGGREGATION[1]

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The results of a previous investigation (Sussman, 1951), indicate that the complex sequence of development displayed by the Dictyosteliaceae can occur in a population derived from a single parent cell. Moreover, the progeny of single cells, taken from widely divergent stages of growth and differentiation, retain this capacity. This would imply that, within a clonally isolated population of myxamoebae, the required cellular varieties can arise and become apparent in the necessary proportions and at the correct times so as to insure normal development. One of the interesting aspects of this implication involves the nature of the mechanisms which provide for the origin of the cellular heterogeneity observed.

The aggregation stage of the development was chosen as the subject of an initial approach to this problem. As a result of past investigation, some features of this phenomenon are well understood. A brief summary of the available information follows.

After vegetative proliferation has ceased, the myxamoebae, heretofore randomly distributed, elongate and become radially oriented. They move toward the center of orientation, at first as individuals and later in streams which become progressively larger and more ramified. In this manner, a rounded mass of cells is built up at the aggregative center. Ultimately the aggregate assumes the typical appearance of a pseudoplasmodium.

Studies by Raper (1941) indicate a complete dissociation between vegetative growth and the onset of aggregation. The results of Bonner (1947) suggest that the process occurs as the result of a response of the outlying cells to the production, by individuals at the aggregative center, of a diffusible substance or substances which Bonner has named "acrasin." Other work by Raper (1940a) indicates that the attracting substances may be specific since cell mixtures of different species will, in many cases, form entirely separate aggregation figures.

Consideration of this phenomenon suggests several pertinent questions which are capable of experimental elucidation.

(a) Is the number of aggregative centers formed a function of the number of cells in the population?

(b) If such a relationship does indeed exist, does it remain constant during the course of growth and differentiation of the population?

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(c) Is the capacity to initiate aggregation possessed by all the myxamoebae or by only a part of the population?

(d) Can a single cell initiate the formation of a center?

The present communication summarizes the salient results achieved during an attempt to answer these questions. The results indicate that the number of aggregative centers is both a function of the number of cells and of the population density. The number of centers/cell at the optimal population densities was determined for two species and was found to be constant for cells at the developmental stages immediately prior and subsequent to aggregations. Under the conditions employed the capacity to initiate center formation is not omnipresent but is possessed by only a small portion of the population. Finally, the results offer some support to the contention that aggregation centers are initiated by single cells.

METHODS

A. The strains employed and their preparation for experiment

Single clone isolates of two species of Dictyosteliaceae, *D. discoideum,* strain NC-4 and *D. purpureum,* strain V-1, were employed in this investigation. Stocks were carried by plating with *Aerobacter aerogenes* on a previously specified medium (Sussman, 1951). Three to five spore masses were picked from a mature culture and mixed with a few drops of a 24–48 hour culture of *A. aerogenes.* The mixture was spread and the plates were incubated at 22° C. This inoculum requires between 44 and 50 hours to grow to maximal number and dispose of almost all of the bacteria. Subsequently, the myxamoebae aggregate and form fruiting structures.

To prepare the cells for experiment, cultures were washed off the plates with cold distilled water and the resulting suspension centrifuged five minutes at 1000–1200 rpm. in an International refrigerated centrifuge. This speed and time are sufficient to free the myxamoebae from most of the occluded bacteria. After three more washes in distilled water, the cells were suspended in either water or a salt solution devised by Bonner (1947).

B. Direct cell counts and surface density determinations

Direct counts were made with the aid of a Levy counting chamber. At least six replicate samples of each suspension were surveyed. The errors involved were found to be less than the fluctuations due to random sampling.

Measurements of surface density of cells on aggregation plates were made under low power using a Sedgewick ocular grid which had previously been calibrated with a stage micrometer. Except where later specified, the myxamoebae did not clump appreciably and the densities could be adequately controlled.

C. Medium for aggregation experiments

All studies of aggregation were carried out on plates containing a 2% washed agar-distilled water medium, hereafter designated as minimal agar. The agar was washed 5 to 10 times and dried in a vacuum desiccator before use.

RESULTS

A. The ability of washed myxamoebae to aggregate and form fruiting structures

In his studies of the nature of the aggregative stimulus, Bonner (1947) had used myxamoebae washed free of bacteria and suspended in a salt solution. Aliquots of the suspension were dispensed in Syracuse dishes. The cells settled at the glass-water interface and, after about 16 hours, began to aggregate. Eventually, typical pseudoplasmodia were formed and at this point the morphogenetic process stopped.

For the purpose of the present investigation, a system was required which would permit accurate counts of the number of aggregative centers and precise control over population number and density. Preliminary experiments using the previously described procedure suggested that it could not completely satisfy these criteria. In devising an alternative method of study, use was made of an observation by Raper (1940b) that aggregating myxamoebae could be transferred to fresh nutrient agar plates whereupon the aggregation continued. A washed agar-distilled water substratum was substituted for the complete medium in order to suppress the growth of occluded bacteria and thus to prevent growth of the myxamoebae. Under these conditions, washed cells remained constant in number, aggregated, formed pseudoplasmodia and eventually complete and typical fruiting structures. Accurate counts of the numbers of aggregation centers were possible and adequate control over population size and density was comparatively easy to achieve.

B. The dependence of the number of aggregation centers formed upon the population size and density

Preliminary experiments were performed in which different numbers of cells were homogeneously distributed over identical areas of agar surface and, subsequent to aggregation, the number of centers counted. The results indicated a complex relationship between the number of centers and the number of cells. For very large populations, the number of centers was largely independent of the population size. For very small populations, the number of centers appeared to vary exponentially with the population size.

Clearly, a change in the total population number, distributed over a constant area must also involve a change in the population density. The possibility was raised that the number of aggregation centers produced by a group of cells is a function both of the total quantity of cells and their surface density. It therefore became necessary to elucidate the effect of the latter condition before one might hope to understand the effect of the former.

The relations between the number of centers and the population density was determined in the following way. A series of dilutions was prepared from a suspension of washed myxamoebae in Bonner's salt solution. An aliquot of each dilution was chosen such that all the aliquots contained the same number of cells. A typical protocol is given below:

No. cells/cc. of dilution	Vol. of aliquot chosen	Total no. of cells per aliquot
5×10^6	0.02 cc.	1×10^5
2×10^6	0.05 cc.	1×10^5
1×10^6	0.10 cc.	1×10^5
5×10^5	0.20 cc.	1×10^5

These aliquots were then dispensed in replicate on minimal agar plates. Since the surface area covered by an aliquot is a function of the volume delivered, the number of cells per unit area could be varied while the total population remained constant.

Trial experiments were run in order to assay the reproducibility and reliability of the procedure. It was found that the surface area covered by an aliquot is directly proportional to the volume delivered within the range employed. The number of cells/mm.^{2} of surface was determined, (a) by direct count under low power using a Sedgewick ocular grid and (b) by calculation using the known numbers of cells and measurements of the areas covered. The two procedures provided calibration data which are in excellent agreement.

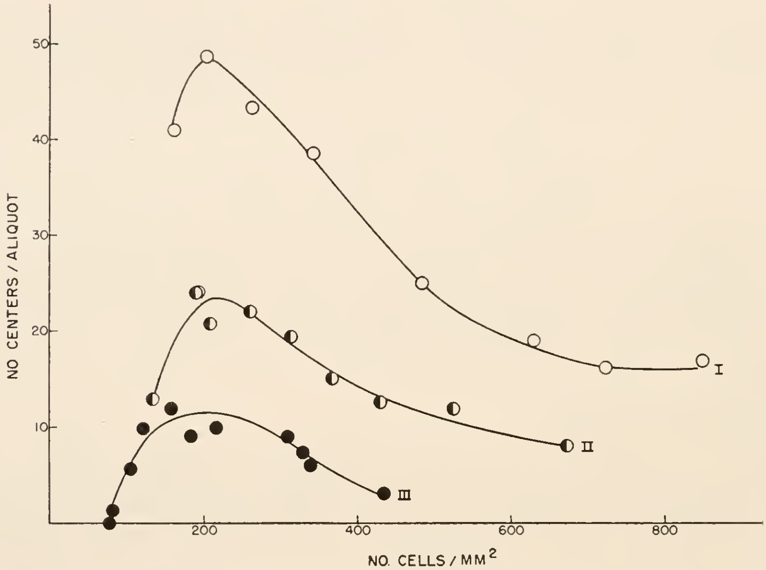

FIGURE 1. Aggregative center formation by *D. discoideum* as a function of population size and density. Aliquots of myxamoebae suspensions were placed on minimal agar plates and were designed to contain a constant number of cells dispersed at different population densities. After incubation, the numbers of centers per aliquot were determined. Curve I represents the data obtained for a population size of 1.0×10^5 cells per aliquot, curve II for 5.0×10^4 cells/aliquot, and curve III for 2.5×10^4 cells/aliquot.

After plates had been seeded in this manner, they were incubated at 22° C. with the covers ajar. Within a short time the fluid was absorbed by the agar and the covers were replaced. Determinations of population density at this time revealed no significant changes due to drying. After about 12 hours, aggregation began. Subsequently pseudoplasmodia were formed, one from each aggregation center, and these later gave rise to complete sorocarps. Counts of the numbers of centers were usually made at 16, 24 and again at 36 hours. Each aliquot was replicated between four and eight times and the means were calculated. The variance was low enough to assure the significance of the values obtained.

Figure 1 summarizes the results of a number of experiments with 44-hour cultures of *D. discoideum*. The cells were harvested immediately prior to the normal occurrence of aggregation. Each point on the curves represents the mean of at least 12 replicate determinations. Three different total population levels were used.

The curves indicate that at very high population densities, the center-forming cells appear to interact competitively resulting in the suppression of a portion of them; the remaining centers are proportionately larger than those at lower population densities. At very low densities, the limit of activity of the aggregation stimulus seems to be reached so that the number of centers falls to zero. At a density of about 200 cells/mm.², no limitation is apparent and the number of centers is maximal.

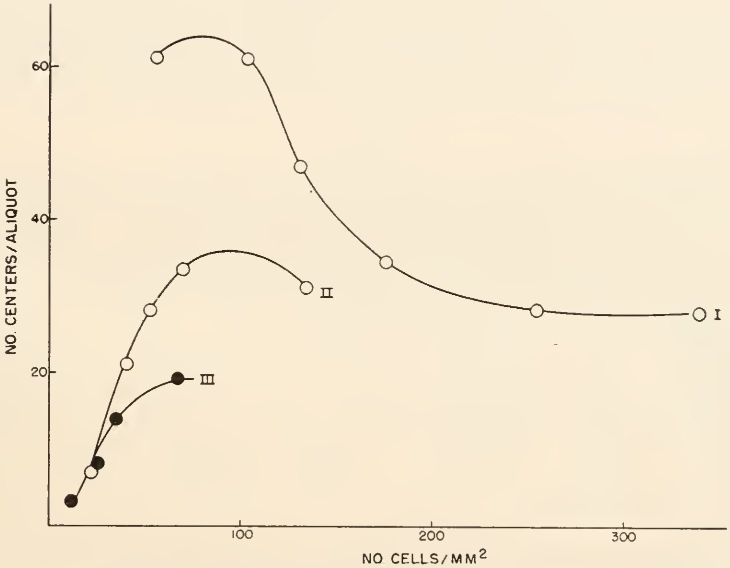

FIGURE 2. Aggregative center formation by *D. purpureum* as a function of population size and density. Aliquots of myxamoebae suspensions were placed on minimal agar plates and were designed to contain a constant total number of cells dispersed at different population densities. After incubation, the numbers of centers/aliquot were determined. Curve I represents the data obtained for a population size of 2.5×10^4 cells/aliquot, curve II for 1.0×10^4 cells/aliquot, and curve III for 5.0×10^3 cells/aliquot.

In the region of this optimal density, the number of centers was found to be directly proportional to the total number of cells present. Calculation of the number of centers/cell within this region revealed the following: for 1×10^5 cells, the number of centers/cell was 4.85×10^{-4}; for 5×10^4 cells this figure was 4.70×10^{-4}; and for 2.5×10^4 cells a value of 4.65×10^{-4} was obtained. The mean value was $4.73 \times 10^{-4} \pm 0.15 \times 10^{-4}$ (standard deviation), equivalent to one center for about 2100 cells.

A similar set of experiments was performed with washed cells from 50-hour cultures of *D. purpureum*, harvested immediately before aggregation. Figure 2 summarizes the results obtained. The number of centers/cell was found to be much higher in this species than in *D. discoideum*, the mean value amounting to

$3.3 \times 10^{-3} \pm 0.5 \times 10^{-3}$. This corresponds to a distribution of one aggregation center for about 300 cells.

C. The number of aggregation centers among cells which already have aggregated

In order to determine if the number of centers/cell was different in a population which had already aggregated, 55–60-hour cultures of *D. discoideum* were harvested, washed, and used for the experiment previously described. These cells had already aggregated or were in the process of doing so. Such cells have great tendency to clump, a condition which could be partially alleviated by the use of distilled water as the suspending medium. Even so, appreciable clumping remained and the results were somewhat erratic. The number of centers/cell for three population levels, 1.0×10^5, 5.0×10^4, and 2.5×10^4 cells, was determined. The mean value was 4.3×10^{-4}. It therefore appears that the aggregation process does not alter the distribution of center-forming capacity in the population.

TABLE I

Center formation by mixtures of young cells harvested before aggregation and old cells harvested after aggregation. Each value represents the mean of four replicate determinations. See text for details of experimental procedure

No. cells per drop	% young cells in population	Mean no. centers/drop at optimal population density	Mean no. centers per cell
1.8×10^5	50	77	4.3×10^{-4}
1.8×10^5	90	93	5.2×10^{-4}
1.8×10^5	99	94	5.2×10^{-4}
1.8×10^5	99.99	76	4.3×10^{-4}
1.8×10^5	100	82	4.6×10^{-4}
		Mean of means 84	4.7×10^{-4}

The possibility remained, however, that this value is constant not because more cells had not attained the ability to initiate center formation but rather because the remainder of the population could not respond to the stimulus imposed. In order to rule out this possibility determinations were made of the number of centers/cell of a series of mixtures of old cells (55–60 hours) and young cells (44 hours). Table I shows the results obtained. Regardless of the proportion of young and old cells, the number of centers formed did not differ beyond experimental error. The similarity was evident for each of the population densities employed.

D. The populational distribution of center-initiating capacity

Under conditions where the degree of cellular dispersion does not limit the initiation of aggregative centers, the number of centers which are formed was found to be directly proportional to the number of cells present. At least two interpretations may be raised to explain this fact.

(a) All individuals in the population are capable of initiating aggregation, acting either singly or in association. However, the realization of this potency does not occur simultaneously in all individuals but rather is distributed in time. Thus, the small proportion of cells which attained this capacity first would be the only ones to initiate centers. The rest of the population, attaining this ability at a later time, would already have been attracted to previously established centers and so could not inform the observer of their acquisition.

(b) The formation of a center depends upon the presence in the population of a single cell or group of cells which is uniquely constituted so as to be able to initiate the formation of the center.

A distinction between the alternatives was made by examining the distribution of center-forming capacity within very small population samples at population densities previously shown to be sufficient to permit the expression of aggregating ability. Were alternative (a) valid, one might anticipate that at least one center of aggregation would appear in every sample even though the times of appearance might differ. In contrast, the validity of (b) would be evidenced by the fact that, for small enough population samples, not every one would produce a center despite the fact that the population density would be great enough to enable the initiating mechanism, if present, to operate. In fact, under the proper conditions the distribution of centers should be in accord with the Poisson series. Thus, the proportion of samples containing 0, 1, 2 and 3 aggregative centers would be given, respectively, by the expression, e^{-m}, me^{-m}, $m^2e^{-m}/2!$, and $m^3e^{-m}/3!$, where m is the mean number of centers per sample.

TABLE II

Population distribution of center-forming capacity. The mean number of centers/cell was calculated by solving for m, the first term of the Poisson expression, $P_o = e^{-m}$, where P_o is the fraction of drops containing no centers and m is the average number of centers/drop. See text for details of experimental procedure

No. cells per drop	No. drops examined	Fraction of drops containing:				Mean no. centers/cell
		No centers	1 center	2 centers	3 centers	
2100	85	0.42	0.50	0.07	0.012	4.1×10^{-4}
1025	91	0.626	0.32	0.044	0.011	4.6×10^{-4}
900	123	0.69	0.285	0.025	0.0	4.1×10^{-4}

Suspensions of washed 44-hour *D. discoideum* were prepared and replicate, direct cell counts were made in the Levy chamber. A Cenco machined loop of 1.0 mm. inside diameter was used to deposit drops of the suspension on minimal agar. The fluid was quickly absorbed leaving the cells homogeneously distributed. The average volume of the drops delivered by the loop was found in trial experiments [2] to be 1.1×10^{-4} cc. $\pm$ 0.1×10^{-4}. Surface density measurements were made as before with a Sedgewick ocular grid and demonstrated the duplicability of the method. The variances of the determinations were in all cases approximately equal to the means, indicating that the only significant error is that incurred by random sampling.

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[2] Very dilute suspensions of myxamoebae were dispensed with the loop. The mean numbers of cells deposited were determined by direct count under low power. The original suspensions were counted with a Levy chamber. From the two sets of data, the volume delivered by the loop could be calculated.

After 16, 24 and 48 hours incubation, each spot of cells on the agar was examined under a dissecting microscope for the presence of aggregations and fruiting structures. At the end of this time, it was found that not all of the spots contained centers of aggregation. Moreover, all the centers which appeared did so before 24 hours. Table II summarizes the data obtained from three experiments in which samples of 900, 1025 and 2100 cells were used. The last column on the right contains calculations of the number of centers/cell. These were obtained by solving the first term of the Poisson expression, $P_0 = e^{-m}$, where P_0 is the proportion of samples containing no centers and m is the mean number of centers per sample. The average number of centers per cell was found to be 4.3×10^{-4}. This compares well with the figure, 4.73×10^{-4}, obtained by the procedure described in Section B.

One may ask whether the suppression of center formation by high population densities might not increase the proportion of samples displaying no centers. That this is not the case can be seen by comparison between the data obtained with 900 cells and with 2100 cells. In the latter samples the population density was more than twice as great as in the former. Yet, despite the greater cell density, the proportion of samples displaying no centers was much smaller. It must be noted in this connection that high cell densities do not inhibit all center formation *per se*. From the results summarized in Figure 1, it would appear that when the population density is high, center initiators are close enough to compete for aggregatee cells and, as a result of this competition, some prospective centers are incorporated by their neighbors. In no case has the existence of a high population density led to the suppression of all centers but merely has acted to reduce the total number. Thus one might expect that in the small population samples at high cell densities, the presence of one initiator would lead to the production of a center but where two or more initiators were present in a single sample, the number of centers formed might be less than the number of initiators. Were this condition to exist, the P_0 values would provide accurate reflections of the distribution of initiators but the

TABLE III

Comparison between the observed and expected distributions of aggregative centers. See text for details

No. cells/sample	2100		1025		900	
m	0.86		0.47		0.37	
Total samples	85		91		123	
No. with 0 centers	36		57		85	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
No. with 1 center	42	30.9	29	26.6	35	31.4
No. with 2 centers	6	13.3	4	6.2	3	5.8
No. with 3 centers	1	3.4	1	0.96	0	0.71
χ^2	45.6		1.00		2.44	
p	<0.01		0.6		0.3	

P_1, P_2, etc., values would be biased. For this reason, only the P_0 values have been utilized in the calculation of the number of centers/cell.

It must be emphasized that the crucial point of the experiment has to do with whether or not every sample can produce a center. The question of agreement with the Poisson series is of secondary importance, for its purpose is to enable us to calculate the number of centers/cell and thus permit a comparison with the figure derived previously.

A rough estimate of the agreement of the observed data with the Poisson distribution was made in the following way. The values of m, obtained from the P_0 figures, were employed in order to calculate the corresponding P_1, P_2 and P_3 values assuming random distribution. Table III compares these figures with those obtained experimentally. The accompanying chi-square estimates indicate that, for the experiments using 900 and 1025 cells/sample, the differences between observed and expected results should be exceeded by chance 30 and 60% of the time, respectively. For 2100 cells/sample, the agreement is very poor. Here, the biasing of the P_1, P_2, etc., values, anticipated previously, was encountered. This is not surprising in view of the fact that the population density of these samples was between 500 and 600 cells/mm.^{2}.

It appears that more efficient utilization of the data might be accomplished by making use of the P_1, P_2, etc., values from the experiments where these values were not biased. However, it is felt that the precision required in order to obtain a definitive answer to the question posed does not warrant this refinement.

Discussion

The reported findings indicate that, by the end of the growth period, a clonally derived population of myxamoebae exhibits a condition of heterogeneity with respect to the ability to initiate aggregation. The question of whether this initiation is contrived by a single cell or by groups of cells acting in concert cannot be answered conclusively from the data at hand. However, two considerations point to the likelihood that the former interpretation is correct.

(a) Microscopic observation of the process reveals that at the beginning of aggregation, the cells elongate and become radially oriented. If the population density is small, it generally may be observed that the center of orientation is occupied by very few cells and often merely by one.

(b) The distribution of aggregative centers among small, replicate samples of a population indicates a constant value for the number of centers per cell despite the fact that in these experiments the mean number of cells per sample was varied and therefore the population density was not held constant. Yet, it is clear that, were a group of cells to cooperate in the initiation of a center, an increase in density would encourage center initiation. In the experiments described, however, increases in density did not affect the proportions of samples with no centers but merely acted to suppress the formation of additional centers in samples which already possessed one or more.

One possible method of distinction would appear to lie in the examination of center formation by small numbers of wild type myxamoebae when mixed with "aggregateless" mutants, capable of responding to the initiating mechanism but not of producing it. A number of aggregateless variants have been isolated and are being investigated with this end in view.

One may inquire into the genetic mechanism by which the aggregators and aggregatees arise. Previous findings (Sussman, 1951) have indicated that the differentiation observed could equally well arise via stable genic mutation and appropriate selection pressures, segregation or zygote formation within the clone, or by easily reversible genic or cytoplasmic modifications. At present, it does not appear that the results reported here can be construed to support or militate against any of these possibilities. However, it is hoped that with the information now at hand, a fruitful examination of this question will be possible.

The relation between the center-initiating mechanism and the production of acrasin, the chemotactic principle, may be examined in the light of the reported results. Observation of the aggregative process (Bonner, 1944) has indicate that the myxamoebae at the periphery of the aggregation pattern do not in all cases move radially toward the center but often migrate toward previously established streams of aggregating amoebae before taking up a radial direction. This would indicate that the production of acrasin is not a property possessed only by individuals at the aggregating center but also can be accomplished by outlying cells. It is therefore likely that the capacity to initiate a center of aggregation is not identical with the ability to produce acrasin. One may speculate that the center-initiating agency may supply a co-factor necessary to the production of acrasin and thereby exert its effect.

SUMMARY

1. Examination of the aggregation stage in the development of the Dictyosteliaceae has revealed that the number of aggregative centers formed is a function of the population size and density. At population densities which do not limit the formation of centers, the number of such centers is proportional to the number of myxamoebae present. For *D. discoideum,* the number of centers/cell was found to be 4.73×10^{-4} and for *D. purpureum,* 3.3×10^{-3}. These values correspond to one aggregation for about 2100 and 300 cells, respectively. The values remain constant throughout the aggregation process.

2. Analysis of the distribution of centers within small, replicate, population samples of myxamoebae indicates that the capacity for center initiation is possessed by only a small proportion of cells. The values for the number of centers/cell achieved by this method agreed well with those previously cited.

3. The implications of these findings are discussed.

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STUDIES ON SHELL FORMATION. I. MEASUREMENT OF THE RATE OF SHELL FORMATION USING Ca^{45} [1, 2]

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Shell growth is commonly measured in ecological and conservation studies as a linear increase occurring in one or a few weeks, or during a shorter period if growth is very rapid. For such studies measurements of growth increments of the order of one millimeter are usually sufficiently sensitive. For the experimental analysis of shell formation, the availability of a method of a different order of sensitivity would be distinctly advantageous and at the same time would make possible new approaches to the study of shell growth. The extreme sensitivity of radioactivity measurements suggested the use of radioisotopes. The determination of the rate at which Ca^{45} is incorporated into growing shell should give a measure of the amount of mineral substance deposited during shorter intervals than would be otherwise possible. The isotope method would also permit for the first time the quantitative measurement of mineral deposition in localized areas of the inner shell surface.

In the present series of studies the radioisotope method has been utilized in the analysis of several phases of shell formation. These include: (1) the quantitative measurement of calcium deposition; (2) rate of transfer of calcium through the mantle; (3) the distribution of inorganic and organic material deposited on the inner shell surface as studied by autoradiographic and histochemical methods; and (4) the exchange of calcium between shell and sea water in the absence of living tissues. This study deals with the first of these problems and is specifically concerned with the examination of the suitability of the isotope method for measuring calcium deposition in the living oyster under standardized conditions. These experiments demonstrate that the isotope method can be used to measure the deposition of calcium and that growth increments occurring in four hours are measureable.

METHODS

Oysters (*Crassostrea virginica* Gmelin[3]) were collected from Bogue Sound near Beaufort, N. C., and kept below low tide level at the laboratory until ready for use. Those to be used for a particular experiment were matched approximately as to size (ca. 8–9 cm. in length) and the surfaces scrubbed free of foreign matter. After drying briefly they were coated with fingernail polish to prevent uptake of Ca^{45} by

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[3] Previously *Ostrea virginica*. Re-named by Gunter (1950).

the outer surface of the shell.[4] The coated oysters were kept below low tide level for several hours or overnight to remove all traces of the solvent.

Single oysters were placed in one liter of sea water in large flat glass dishes and shell movements were recorded on a kymograph throughout the experimental period. Such records provided data on periods of closure as well as indicating normalcy of shell movements. If shell movements were normal, the sea water was renewed and Ca^{45} added to give an activity of 6×10^5 counts per minute per liter of sea water. The Ca^{45} was of high specific activity or carrier-free so that the calcium content of the sea water was not increased appreciably. The radioactive sea water was renewed every four hours. The temperature range was 21.2 to 25.4° C. for the entire period of study though approximately constant for each experiment. Sea water salinity was approximately 35 parts per thousand.

After intervals of 8, 16 and 24 hours, oysters were removed from the sea water containing Ca^{45} and the shells prepared for determinations of the radioactivity of the inner shell surface. The mantle was removed and the region of muscle attachment carefully scraped free of material. The shell was then rinsed thoroughly with a fine stream of distilled water from a wash bottle. Previous experiments had shown that this treatment was sufficient to remove all adhering material which might be radioactive and was as effective as scrubbing with a stiff brush. The radioactivity was measured for circular areas of 6.2 cm.^{2} in the following regions: extreme posterior end; center which included a portion of the site of muscle attachment; and the anterior end immediately posterior to the hinge. Essentially constant geometry was maintained by means of a special holder in which the shell was held close to the window of the counting tube for the measurements just mentioned or against a brass plate with a circular aperture of 1 cm^2. for more exact localization of radioactivity. Because of geometrical difficulties with the cupped left valve, measurements were confined to the less curved right valve. A thin window counting tube (1.9 mg./cm^2.) was used.

Carefully cleaned right shells were included in all experimental solutions in order to obtain an estimate of the radioactivity of the shell resulting from exchange between sea water and shell as contrasted with deposition by the living oyster. The values for the living oyster were corrected for exchange in each instance.

RESULTS

Figure 1 shows the Ca^{45} deposited after various intervals in the posterior region of shell of 60 oysters. The mean Ca^{45} deposition was directly proportional to the period of exposure to the isotope. Marked individual variation is apparent and will be discussed later. Graphs of the center and anterior regions gave a similar picture but differed quantitatively from the posterior region. Calcium deposition could be measured after four hours in radioactive sea water under these conditions, as suggested in Figure 1 and as demonstrated in experiments not reported here. With higher concentrations of Ca^{45}, deposition would be measurable after still shorter intervals.

The deposition rate was not uniform over the shell surface; the relative mean depositions after 24 hours for the three regions measured were: posterior 1.0, center 0.55 and anterior 0.58. The regional differences were investigated further by making measurements of Ca^{45} activity on several circular areas of 1 cm.^{2} (Fig. 2). The positions of the columns indicate the regions for which radioactivity was determined and the height gives the relative deposition. The non-uniformity of rate of deposition measured in small areas is more marked than with the larger areas mentioned above. This general pattern, which represents the means from 8 oysters, also characterized the individual shells. The main features of the regional differences are the following: (1) The differences in deposition in adjacent small areas may be great. And the pattern of rates obtained will accordingly depend upon the size of the area included in the individual measurements, as may be seen by comparing the values for the three larger areas with those in Figure 2. (2) The rate of deposition in the posterior portion was relatively high, although the thin posterior edge was low. (3) The portions near the periphery of the shell exhibited a higher rate than the interior. An autoradiographic study, which will more exactly define the pattern of deposition, is now in progress.

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[4] P^{32} is taken up by nail polish though not by a coating of wax.

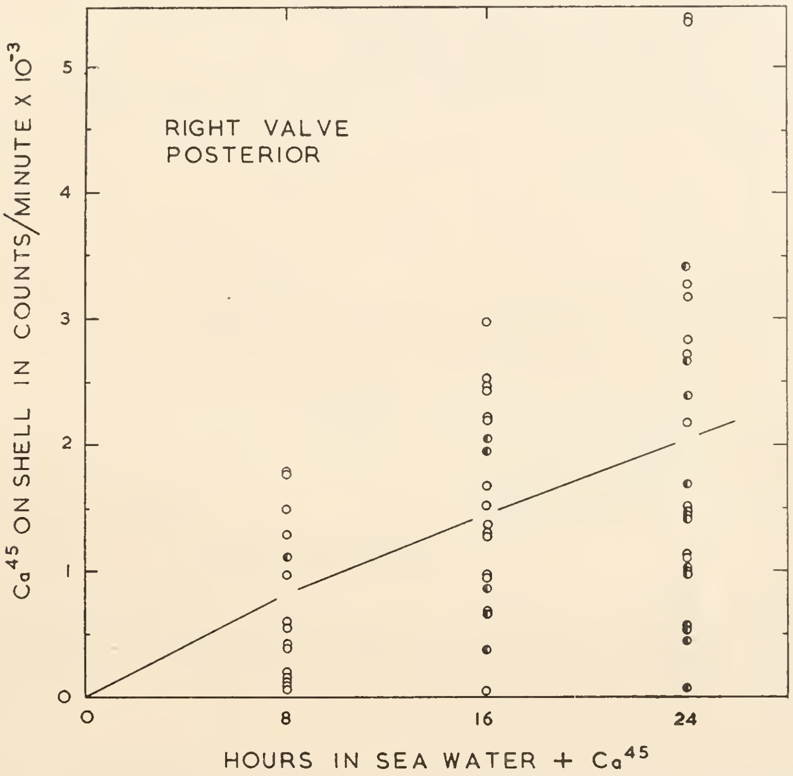

FIGURE 1. Ca^{45} deposition by the living oyster. Each point represents the radioactivity of a circular area of 6.2 cm.^{2} at the extreme posterior end on the inner surface of the right valve of one oyster. The line connects the mean values for Ca^{45} deposition for each interval. The half closed circles represent Ca^{45} deposited by oysters which remained closed for more than 25% of the experimental period.

An approximation of the weight of calcium deposited can be made by comparing the radioactivity of the sea water and the shell. Such an estimate becomes possible when the following conditions are operative:

1. *The measured radioactivity of the shell is directly proportional to the total Ca^{45} deposited in a given interval.* This will be the case provided the layer of new material is sufficiently thin that the radiation from the calcium first laid down is not absorbed by the salts and conchiolin deposited later. The estimated amounts of $CaCO_3$ deposited (see method below) in the intervals used are such that the factor of self-absorption can probably be neglected (Comar *et al.*, 1951) except with the higher values found with 24 hours exposure [5] (Fig. 1).

2. *The calcium being deposited is of the same specific activity as the sea water.* Other studies (Jodrey, 1952) have shown that the Ca^{45} in the mantle has reached equilibrium in about one hour. The fact that the mean radioactivity is a straight line function of time (Fig. 1) also indicates that equilibrium is attained relatively quickly.

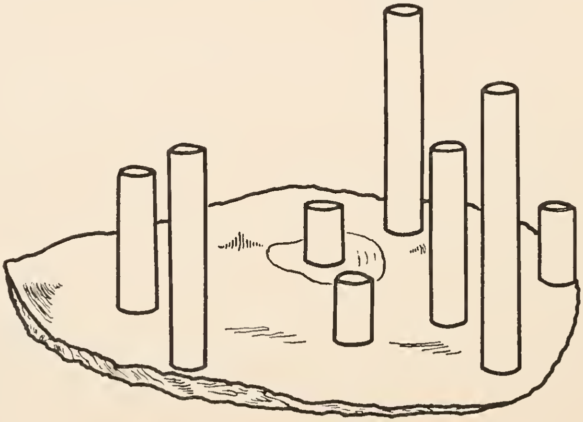

FIGURE 2. Distribution of newly deposited calcium on the inner surface of shell. Ca^{45} deposited after 24 hours was measured on several one cm.^{2} regions of the shell as indicated by the position of the columns. The height of the columns shows the relative amounts of Ca^{45} deposited. The site of the muscle attachment may be taken as 1. The figures represent average values for 8 shells.

3. *The difference in backscatter in measurements of radioactivity of sea water and shell is known.* Radioactivity measurements of the sea water were made on copper planchets whereas measurements of shell were made against the $CaCO_3$ of the shell itself. The higher atomic number of copper as compared with calcium would give an increased backscatter and an underestimation of the calcium deposition of the order of 8 or 9% (Glendennin and Solomon, 1950).

4. *The specific activity of the sea water is not appreciably changed by unlabeled calcium from the tissues and body fluids.* Error from this cause would be negligible

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[5] With the mean deposition rate of 9.0×10^{-3} mg. $CaCO_3$/cm.^{2}/hr. found in the posterior region, the limiting value of approximately 0.5 mg./cm.^{2} would be reached in about 56 hours. For the shells showing most active deposition (Fig. 1) the limiting value has already been exceeded slightly in 24 hours.

because of different orders of magnitude (ca. 400 mg. Ca per liter of sea water and ca. 7 mg. Ca in the oyster (Sherman, 1947)). The renewal of the radioactive sea water every four hours would further minimize this factor.

Under the conditions defined above the calcium deposition can be calculated from the following relation:

$$D = \frac{A_s}{A_w} \times C,$$

where D is mg. Ca deposited cm.^{2}, A_s is counts/min./cm.^{2} on shell, A_w is counts/min./liter sea water, and C is mg. Ca/liter sea water.

Using this relationship without further correction, the mean amount of calcium deposited was calculated to be 9.0×10^{-3} mg./cm.^{2}/hr. for the posterior region of the right valve and 5.2×10^{-3} mg. for a similar area in the anterior region. If we assume for purposes of a rough estimate an average value of 7×10^{-3} mg. Ca/cm.^{2}/hr. for both valves, an oyster of 8.5 cm. in length with an internal shell surface of 70 cm.^{2} would deposit 0.92 gm. of $CaCO_3$ in a month under these particular conditions. If the $CaCO_3$ constituted about 90% of the shell weight, the total weight gain would be approximately one gram. Because of difference in species and unknown differences in conditions, a comparison with the results of others (Havinga, 1928; Masumoto *et al.*, 1934; Galtsoff, 1934) on shell growth in natural waters is scarcely possible.

The rate of deposition differed widely in different individuals (Fig. 1) as previously mentioned, even though oysters were of approximately the same size collected from the same site, treated identically, and exposed to radioactive sea water simultaneously. However, since a size range, although small, did exist, the calcium deposition for each period of exposure has been examined with respect to various parameters of shell size by plotting the amount deposited against length, width, area, weight, and average thickness (area/weight). The scatter of points in each case indicated the absence of significant correlation. It should be pointed out that inasmuch as individuals used were selected for similarity of size, the present findings in no way indicate an absence of correlation between rate of deposition and size for a greater size range. Orton (1935) has shown a negative correlation between relative growth rate and size in the English oyster, while a low degree of correlation between rate of growth and size and weight of oysters has been reported by Havinga (1928).

The erosion of the inner surface which takes place when mollusks remain closed for extended periods suggested the possibility of removal of previously deposited calcium during the relatively long periods of closure which occurred in some individuals. Also, if deposition continued during closure, it might proceed at a different rate. To investigate the effect of closure, the time that each of the 60 oysters remained closed was determined from the kymograph records of shell movements. The deposition in individuals which remained closed for more than 25% of the experimental period appeared to be about the same as those which remained open for a longer time except for the 24-hour period where less deposition took place in the posterior region. (See Fig. 1.)

The observed variability in growth rate is not peculiar to the conditions of these experiments, for great variability is also the common finding in oysters growing in natural waters (Havinga, 1928; Loosanoff and Nomejko, 1949; Butler, 1952).

Discussion

The results presented indicate the feasibility of the use of Ca^{45} in studies of shell growth in the oyster. The use of radioisotopes offers two distinct advantages over conventional methods: (1) Extremely small growth increments such as those occurring in a few hours can be measured. (2) Differences in rate of deposition in localized areas can be determined. For comparative measurements of shell growth the radioactivity of a single area such as the posterior region can be used as an index of growth. Because of localized differences in deposition over the surface, total growth can be determined only by measurement of the radioactivity of the whole surface.

The isotope method for studying shell growth also imposes definite limitations. The experimental periods must be short in the case of rapid growth; otherwise absorption by layers overlying the material deposited previously will interfere with the measurement of the soft β radiation of the Ca^{45}. . Because of the expense of isotopes and for practical considerations, the volumes of the radioactive solutions will normally be small, thus placing restrictions on the type of studies which can be carried out conveniently. Moreover, particular consideration must be given to the maintenance of a favorable environmental situation with small volumes. As judged by normal shell movements this requirement appeared to be reasonably well fulfilled in the present studies by placing a single oyster in one liter of sea water in a shallow dish and changing the sea water every four hours. Radiation injury is probably not a complicating factor inasmuch as the radioactivity of the medium can be kept relatively low. An obvious limitation of the method is that successive measurements of growth cannot be carried out on a single individual.

In addition to the active deposition of Ca^{45} by the oyster, a shell will become radioactive by an exchange of atoms of the shell for those in the surrounding fluid. As a result of this process of exchange a shell carefully cleaned of all tissue will take up increasing amounts of Ca^{45} as it remains in radioactive sea water. The extent of exchange in the shell of the living oyster cannot be estimated precisely without knowing the calcium concentration and specific activity of the fluid in contact with the inner shell surface; but on the assumption that these values are not very different from sea water (Hayes and Pelluet, 1947), an approximation can be made from the radioactivity of cleaned shells placed in the radioactive sea water with the experimental oysters. For the posterior region the exchange so measured was about 1% of the mean and 0.5% of the maximum amount deposited by the living oyster during 24 hours.[6] It is apparent that exchange offers no serious obstacle to the measurement of shell growth.

The non-uniform distribution of newly deposited calcium over the inner shell surface indicated differences in deposition rates which must eventually be correlated with cell structure and mechanisms in localized regions of the mantle. The low

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[6] Exchange in shell will be considered in detail in a separate publication.

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rate of deposition in the thin posterior shell margin is especially interesting. This finding does not in itself mean a lower growth rate but perhaps a high organic : inorganic ratio of material deposited. Orton (1928), working with English oysters, mentioned that this thin shell margin hardens later in the season; and Loosanoff and Nomejko (1949) found that the hardness and brittleness of the new growth of Long Island Sound oysters is greater as the environmental temperature increases. These findings strongly suggest that the type of material laid down by the mantle shifts with certain environmental changes. In view of this, the pattern of deposition found in this study might be quite different under other conditions. This could be studied by exposing oysters to Ca^{45} under the desired conditions and making an autoradiographic study of the inner shell surface.

Summary

1. The rate of shell formation in the oyster *Crassostrea virginica* Gmelin has been determined by measurements of Ca^{45} deposited by oysters placed in sea water containing Ca^{45}. The experiments were carried out at Beaufort, N. C., during May and June at 21.2 to 25.4° C. Radioactivity measurements were made directly on the inner shell surface.

2. The mean amount of deposition was directly proportional to the time of exposure to Ca^{45} for 8, 16 and 24 hours. Growth increments occurring in four hours were measurable. Individual variation in the rate of deposition was marked.

3. The mean rate of deposition in the posterior region of the shell was approximately twice that in the center and anterior regions. Within a general region local areas may exhibit a still greater difference in rate. From the average radioactivity per unit area of shell surface the total amount of $CaCO_3$ deposited in a given period can be calculated.

4. Shell from which all tissue had been removed became increasingly radioactive in sea water containing Ca^{45} as a result of exchange. The magnitude of exchange was relatively very small as compared with active deposition by the oyster.

5. Advantages and limitations of the isotope method for measurements of growth are discussed.

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ABSTRACTS OF SEMINAR PAPERS

July 1, 1952

An enzymatic study of yolk-platelet lysis. Paul R. Gross.

By means of a photometric method, the lysis of yolk-platelets isolated from amphibian eggs can be followed quantitatively. In this manner, a study was made of the rapid yolk-platelet dissolution which takes place in the presence of divalent cations, particularly calcium.

The lytic reaction was found to be pH dependent, with a sharp optimum near pH 6.0. When temperatures were increased beyond 60° C., the velocity of the reaction fell rapidly, although at 60–70° C., there was no microscopically observable damage to the platelets.

From yolk-platelet suspensions, it was possible to prepare a factor which then induced a more rapid lysis of other suspensions when Ca ions were added. Controls, to which this factor, previously boiled, was added, showed only the minimum reaction velocity for the system. Treatment of the factor with protein precipitants also destroyed its activity. The variation of lytic rate with concentration of this factor was, under certain conditions, linear.

Yolk-platelets may be lysed by hypertonic ($> 0.3\ M$) NaCl or KCl. If this lysate is dialyzed or diluted, there is a precipitation of an amorphous, lipid-containing protein material. This solubility change with respect to Na or K concentration is reversible. When, however, very small amounts of Ca (*e.g.*, $5 \times 10^{-3}\ M$) are added to the precipitate, this is rapidly and *irreversibly* solublized. The Ca-mediated reaction apparently alters the yolk material chemically.

The results of these and other experiments are interpreted as indicating the presence in yolk of an enzyme which is able to lyse the platelet in the presence of small amounts of Ca, Mg or Sr ions. This enzyme is not the phosphoprotein phosphatase first described in yolk by Harris.

The action of heparin preparations on cells and tissues.[1] Alfred B. Chaet.

Heilbrunn has shown that substances which prevent blood clotting, such as heparin, also tend to prevent protoplasmic clotting. To study further the effects of commercial heparin on living cells, it was felt advisable to utilize the frog heart as a test object. In this study kymograph recordings were made of isolated hearts which were perfused with various concentrations of heparin (Hynson, Wescott & Dunning, Lederle, and Upjohn) dissolved in frog Ringer. When concentrations of heparin of 0.4–0.5% were used, a cessation of heart beat was observed. The heart remained in a relaxed state, as might be expected; and the effect was reversible. This effect is due to a substance capable of passing through a dialysis membrane, and therefore with a lower molecular weight than that of ordinary heparin. This relatively small molecule, obtained by dialyzing some commercial heparin preparations, is also capable of inhibiting cell division of fertilized eggs of the clam, *Spisula solidissima,* and of the starfish, *Asterias forbesi.* The inhibitory action of this molecule is lost with time when the preparation is stored at + 6° C., but retains activity for at least 8 days if frozen at − 22° C. The dialyzable fraction becomes metachromatic after remaining in contact with toluidine blue for several hours (heparin produces metachromasia immediately in the presence of toluidine blue). The dialyzed fraction has no effect on the clotting of blood when used immediately after dialysis. However, preliminary experiments indicate that under certain conditions the dialyzable fraction does have an effect.

The relatively small molecule present in some commercial heparin preparations is not phenol and is probably not any one of the other substances used in heparin purification. Indeed, there is evidence to indicate that the dialyzing substance may possibly be a breakdown product of the larger heparin molecule.

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[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

An attempt to influence the survival time of cancerous mice.[1] L. V. HEILBRUNN, ATIDA HALABAN AND W. L. WILSON.

Experiments of Most in our laboratory have shown that when muscle is fatigued it gives off heparin or heparin-like substances (Most, in press, 1953). In view of the fact that heparin prevents cell division, and in view of the fact also that Bullough (1948) found that exercise markedly decreased the rate of mitosis in the skin of mice, we investigated the effect of exercise on the survival time of mice implanted with tumors. We used two types of malignant tumors, and the mice with these tumors were placed in cages containing an exercise wheel. The survival time of these mice was compared with the survival time of similar mice in cages without exercise wheels. Exercise appears to have a favorable effect on the survival time.

JULY 8, 1952

Studies on initiation of cleavage in the frog egg. J. R. SHAVER, S. SUBTELNY AND A. WANIA.

It has previously been shown that injection of centrifugal fractions of frog and mammal tissue homogenates into unfertilized frog eggs will produce parthenogenetic cleavage. A large part of the cleavage-initiating material is associated with particulate elements of the cytoplasm, especially larger granules. The capacity of cytoplasmic granules to initiate cleavage begins to appear in frog embryonic cells at late blastula, reaches a peak exceeding that of granules from adult tissue during a three- to four-hour period at early gastrulation, and decreases somewhat during subsequent developmental stages. Inhibition of development with sodium azide at gastrulation also inhibits the cleavage-initiating capacity of granules isolated from homogenates of such blocked embryos. Reversal of inhibition partially restores activity of the granules. Similar results have been obtained using p-chloromercuribenzoate, an agent known to inhibit the activity of sulfhydryl-containing compounds, the inhibitory effect of which can be reversed by treatment of granules with cysteine. Large cytoplasmic granules prepared from gastrula homogenates and subjected to ultraviolet radiation at 2537 Å lose their cleavage-initiating capacity after a dosage of 3960 ergs/mm.^{2} or more. Some preliminary results indicate the possibility of reactivating granules inhibited in this way with visible light. The results of these studies are interpreted provisionally as indicating: (a) the physiological role of the constituents of the cytoplasmic granules in establishing conditions favorable for initiation of cell-division; (b) the possibility that thromboplastic activity of granules may produce viscosity changes in egg cytoplasm necessary for spindle formation; (c) a quantitative and/or qualitative change in the constitution of cytoplasmic granules during development.

The structure of the "neuromotor apparatus" of Paramecium as revealed by the electron microscope. DOROTHY R. PITELKA AND CHARLES B. METZ.

The "neuromotor apparatus" of Paramecium, as seen with the light microscope, consists of a system of cilia, ciliary basal bodies (kinetosomes) and longitudinal, sub-pellicular fibers (kinetodesma) connecting adjacent basal bodies. In view of the presumed integrative function of these neuromotor fibers and the morphogenetic properties of the basal bodies, a more detailed study of this system seemed desirable. By appropriate technique (ultrasonic dissection of fixed animals) fragments of pellicle with associated basal bodies and neuromotor fibers were obtained for electron microscope study. The neuromotor apparatus of two species, *P. calkinsi* and *P. multimicronucleatum,* was examined. Starving, not dividing, animals were used throughout.

In *P. calkinsi* two basal bodies were associated with each pellicular polygon. Each "neuromotor fiber" consisted of not one fiber but a bundle (3–4) of fine parallel fibrils (approximate diameter 0.1 μ). Each fibril contained a spiral structure with a period of approximately 400 Å.

P. multimicronucleatum proved to be more favorable for a study of the relation of the neuromotor fibers to the basal bodies. In this form one basal body was associated with each pellicular polygon. Again, the "neuromotor fibers" consisted of a bundle of several fine fibrils. Each of these fibrils arose independently from a ciliary basal body and joined the bundle. Each fibril extended for some distance as a member of the bundle and finally tapered to a fine point. Thus, the basic structure of the neuromotor system in *P. multimicronucleatum* is a bundle of fine fibrils. Each fibril arises from a basal body. These fibrils appear to be of constant length and overlap in shingle-like fashion along the bundle.

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[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

JULY 15, 1952

A visible response to stimulation in Spisula eggs. ROBERT DAY ALLEN.[1]

It has been observed that fertilization or artificial activation is followed by changes of shape in the eggs of the surf-clam, *Spisula solidissima.* Slight surface wrinkling results from application of agents which cause high percentages of germinal vesicle breakdown (activation). Some other parthenogenetic agents (heat, cold, urea, sodium and calcium ions) have little activating effect on *Spisula* eggs, but do cause deep surface indentations to appear. These structural changes cannot be induced in the absence of calcium. They are not osmotic effects, although they can be duplicated by removal of water with hypertonic solutions, or return to sea water from hypotonic solutions.

Haematocrit measurements clearly show that eggs with indentations have undergone a decrease in volume of at least 6–8%. This indicates that eggs lose water during their response to stimulation. Water loss (syneresis) in inanimate gels accompanies ageing or increase in rigidity. *Spisula* eggs which have aged 10–16 hours, or have been stimulated in the presence of calcium, show evidence of syneresis. An increased gelation can probably be inferred from the observed loss of water. Because of the brevity of the structural change, a relative viscosity increase could be detected only with prolonged indentations in eggs subjected to short heat treatment.

The frequency of indentations at the vegetal pole suggests contraction by the cytoplasmic gel, because the vegetal region of the egg contains the thickest band of cytoplasm. The properties of the cytoplasm are such that ultracentrifugation produces a ring of negative birefringence around the displaced nucleus. There is no apparent change in this birefringent region after fertilization.

The isolation and chemical characterization of the mitotic apparatus of dividing cells. DANIEL MAZIA AND KATSUMA DAN.

Methods have been developed for the isolation in quantity of the mitotic apparatus of dividing sea urchin eggs (*Strongylocentrotus purpuratus* and *S. franciscanus*). Essential features of the methods are (1) arrest of mitosis by means of 30 per cent ethanol at − 10° C., (2) stabilization of the MA by treatment with dilute H_2O_2, and (3) dispersal of the cytoplasm by dilute Duponol solutions.

The structure of the isolated MA at various stages of mitosis corresponds to the classical cytological descriptions. All of the major elements—nuclei or chromosomes, centrioles, spindles, asters and midbodies—are removed together as a single physical entity. The fibrous elements of the spindles and asters are positively birefringent with respect to their long axes as in the living condition.

A protein fraction has been separated from the isolated MA by solution in alkali and precipitation in the range pH 5–6. The protein accounts for most of the mass of the MA and for about two per cent of all the protein in the egg. Preliminary studies with the analytical ultracentrifuge reveal a single major component of particle weight *ca.* 45,000. There is evidence that the linking of the particles to form the fibrous structures of the MA involves S–S bonding.

Mitotic activity in relation to differentiation in the slime mold, Dictyostelium discoideum. J. T. BONNER. No abstract submitted.

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[1] Public Health Service Fellow, National Cancer Institute.

Electrical method of "sexing" Arbacia and obtaining small quantities of eggs. ETHEL BROWNE HARVEY.

When you pass an electric current through an Arbacia, it sheds its eggs or sperm; a few eggs or a little sperm exude from each of the five gonopores, and the shedding stops immediately when the current is cut off. The procedure is as follows: Place the animal, dorsal (aboral) surface up, in a stender dish small enough to restrict it somewhat and just covered with sea water. *Alternating* current should pass from an ordinary wall socket through a Variac or rheostat to reduce the voltage to 10–15 volts. The electrodes (preferably platinum, as copper is toxic) are put at any two separate places on the shell. If copper electrodes are used, they should be covered with absorbent cotton, frequently changed. When the current is allowed to pass, within a minute the animal will shed; the eggs or sperm come from each gonopore in a fine line, the eggs tending to clump. The best eggs are those taken with a pipette immediately as they exude; these will give 100% fertilization and develop normally. By this method one may easily distinguish males from females by the color of the exuding sperm (white) or eggs (red), and "sex" them, separating males and females for further use. One can also obtain a few eggs at a time without sacrificing the animal. And one can use the same animal repeatedly to carry out experiments on successive days with uniform material.

The reason for the effect is that the walls of the ovary and testis contain smooth muscle cells which cause them to contract on electrical stimulation, thus forcing out the eggs or sperm.

A more elaborate electrical method was described two years ago by Iwata for Mytilus and a Japanese species of sea urchin, *Heliocidaris crassispina*. The simple method described above has been used successfully also for the American sand dollar, *Echinarachnius parma*, and can probably be used for many other animals.

Sperm of Arbacia will keep several days in a refrigerator at about 8° C. if kept concentrated in a small covered stender dish (about 3 cm. diameter). As is well known, sperm when diluted will last only a few hours. When a male is found by electrical stimulation it is best to cut it open and remove the testes as intact as possible for future use. By keeping sperm on hand and obtaining eggs by the electrical method, one can obtain fertilized eggs quickly at any time.

JULY 22, 1952

Further studies of the intercerebralis-cardiacum-allatum system of insects. BERTA SCHARRER.

Certain difficulties regarding the evaluation of the role of the corpus cardiacum in insects can be overcome by considering the organ not as a separate gland of internal secretion, but as a component of a neurosecretory system which is analogous to corresponding organ systems in crustaceans and in vertebrates. The concept that the corpus cardiacum serves as a reservoir for neurosecretory material produced by the pars intercerebralis of the brain and transported to the storage organ via the axons of the neurosecretory cells can be further substantiated by the effects of the interruption of the neurosecretory pathway connecting the brain with the corpora cardiaca. Severance of this pathway (nervi corporis cardiaci) in *Leucophaea maderae* leads to an accumulation of neurosecretory material proximal, and to its depletion distal to the site of operation. This result offers conclusive evidence that in the intact system the neurosecretory substance originating in the pars intercerebralis of the brain is transported via the nervi corporis cardiaci to the corpora cardiaca where it is stored. This interpretation of the morphological observations explains why no acute hormone deficiency seems to occur as a result of the extirpation of the corpora cardiaca.

The neurohypophysis of elasmobranch fishes. ERNST SCHARRER.

Recent investigations indicate that the hormones which can be extracted from the neural lobe of the pituitary gland, are not produced there (Bargmann and Scharrer, 1951). The neural lobe of higher vertebrates consists of glia cells, blood vessels, connective tissue, and nerve terminals. The latter store a protein substance which is elaborated by neurosecretory cells in the hypothalamus and which contains the active (oxytocic, vasopressor, antidiuretic) principles of the neurohypophysis. This concept is supported by observations in elasmobranch fishes in which the neural lobe of the pituitary is supposed to be absent. In fact, the essential components of the neural lobe, namely, the nerve terminals of the preoptico-hypophyseal tracts are present, but they are distributed among the cells of the intermediate lobe. These terminals, like those in higher vertebrates, store the neurosecretory material produced by the cells of the preoptic nucleus. The sum total of these nerve endings should be considered as an equivalent of the neural lobe of vertebrates other than the elasmobranchs, the only difference being that in higher vertebrates the nerve terminals together with blood vessels and supporting tissue form a separate neural lobe.

Balantidial infection in tadpoles as recorded by cine-photomicrography. CARL CASKEY SPEIDEL.[1]

The ciliated protozoon Balantidium is the causative agent for the disease balantidiasis. In man this is accompanied by symptoms resembling those of amebic dysentery.

Balantidia may also cause balantidiasis in tadpoles. These parasites may invade the tissues either by way of the alimentary tube or by way of lesions in the skin. Motion pictures vividly reveal the various histological stages of such infections, both in tadpoles that eventually succumb and in those that recover. Pictures in natural color afford an effective demonstration of balantidia with ingested red blood cells in the circulating blood.

Among the scenes presented are the following: the invasion of the epidermis of the tail through experimentally induced wounds; the movements of balantidia through the subcutaneous tissues; the boring apparatus of the parasites in action; the rapid and conspicuous cytolytic effects caused by mass infections; the destruction and sloughing of epidermal cells; the formation of ulcers; the extravascular phagocytosis by balantidia of extruded erythrocytes in hemorrhagic zones; balantidia in the lymph and blood circulation; the intravascular phagocytosis of erythrocytes; the ingestion of various kinds of tissue fragments; and the multiplication of the parasites by binary fission. In tadpoles destined to recover, each invading balantidium is immobilized and destroyed by a group of leukocytes of the host.

JULY 29, 1952

Sperm agglutinins in the egg jelly of the frogs Rana pipiens Schreber and R. clamitans Latreille. G. S. BERNSTEIN.

Specific interacting substances (fertilizin and antifertilizin) have been obtained from the gametes of many invertebrates, but few vertebrates have been studied in this respect. The purpose of the present work was to determine whether sperm agglutinins were present in the egg jelly of several species of frogs. It was found that the egg water of *Rana clamitans* irreversibly agglutinates *R. clamitans* sperm and loses its agglutinating capacity after three or four days. Sperm agglutinins were not detected in egg water prepared from unovulated (jelly-less) eggs. *R. pipiens* egg water, however, did not agglutinate homologous sperm from September through the May breeding season. This may be due to the relative insolubility of *R. pipiens* egg jelly (the agglutinins are presumably located in the jelly layers), or *R. pipiens* fertilizin is possibly univalent. *R. pipiens* egg jelly is not visibly soluble in pond water, acidified pond water, or in low concentrations (10^{-1} to 10^{-5} M) of NaCl, urea or glycine. When placed in pond water, *R. clamitans* jelly swells to a greater extent than *R. pipiens* jelly. Both *R. pipiens* and *R. clamitans* jellies are soluble in alkali (5% KOH was found to be the best solvent), but the extracts were inactive.

Although these results show that sperm agglutinins are present in the jelly of *R. clamitans* eggs, the presence of agglutinins in *R. pipiens* egg water, as reported by Glaser in 1921, could not be demonstrated.

A function of the salivary glands of the larvae of Drosophila and other flies. G. FRAENKEL.[2]

The puparia of *Drosophila, Phormia regina* and many other flies are usually glued firmly to a surface. During the formation of the puparium, when the pupal contraction is almost finished, a liquid secretion flows out from the mouth and is, by movements of the anterior tip of the body, distributed over the anterior section of the ventral surface. This secretion hardens within a few seconds and effects a firm attachment of the puparium to a substrate. The secretion is protein in nature. The development of the salivary glands has been described by Ross (1939) and Bodenstein (1943). The glands reach their largest size in the prepupa, after feeding has already ceased. Then they show high secretory activity with the product of this secretion finally collecting in the lumen of the gland. After the formation of the puparium all or much of this secretion has disappeared from the lumen. This secretion is of proteinous nature. The secretion in the glands and the glue by which the puparia are fixed to a surface have been studied by paper chromatography and appear to have a similar, if not identical, amino acid composition. Besides, both products contain free lysine and free glucosamine. The formation of this secretion takes place during the period when the salivary gland cells are largest and most favorable for a study of chromosome banding. This function of the salivary glands does not exclude the possibility that at an earlier stage, during feeding, they produce digestive enzymes. However, the puparia of many species of flies are never stuck to a surface and still show some secretory activity in the salivary glands of the prepupa.

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[1] Aided by a grant from the American Cancer Society (Committee on Growth).

[2] In collaboration with V. Brookes and H. Moorefield.

Carotenoid formation by mutant strains of Neurospora crassa. FRANCIS HAXO.

Neurospora crassa normally synthesizes an elaborate mixture of C_{40}-polyenes comprising less than 0.1% of the dried mold. Some 14 components have been identified including acidic xanthophylls, neutral xanthophylls (spirilloxanthin, lycoxanthin, hydroxy-gamma-carotene), carotenes of different degrees of hydrogenation (lycopene, gamma-carotene, beta-carotene, delta-carotene, rhodopurpurin, neurosporene, and a "zeta-carotene-like fraction," actually a mixture of two new carotenes), and colorless C_{40}-polyenes (phytofluene and phytoene).

From among 8 mutant strains which showed an altered polyene composition, four types of mutants have been encountered: (1) specific pigment block, *e.g.*, elimination of the acidic fraction; (2) multiple pigment deficiencies characterized by elimination of xanthophylls and most carotenes, reduced synthesis of hydrogenated polyenes, and a normal phytoene content; (3) carotenoid-less mutants, apparently no carotenoid formed, normal or enhanced phytoene formation; (4) phytoene-less mutants, no phytoene detected, one or more pigments produced in greatly reduced amounts. Although at present the detailed findings cannot be reconciled in a simple biosynthetic scheme, possibly due to the complicating effects of modifier genes, they are consistent with proposals that acidic carotenoids represent terminal stages in carotenoid synthesis and that phytoene is closely related chemically to the well established C_{40}-polyenes. Furthermore, the finding of a phytoene-less mutant capable of forming even a small amount of carotenoid pigment suggests that colorless and colored C_{40}-polyenes may be formed by parallel lines of synthesis from a common precursor. So far, carotenoid deficiencies in Neurospora have not been correlated with any physiological abnormality. The phototropic response characteristic of the wild type strains is also displayed by albino mutants.

AUGUST 5, 1952

Meromyosins, the sub-units of myosin. ANDREW G. SZENT-GYORGYI. No abstract submitted.

Studies on the visco-elastic properties of the anterior byssal retractor muscle of Mytilus edulis. WILLIAM H. JOHNSON AND JOHN F. PERKINS, JR.

Inasmuch as the isometric myogram describes only rate of change of tension with time, additional information regarding the contractile system is obtained by a measurement of viscosity (mechanical resistance) and elasticity of contracting muscles. For example, the myogram does not tell whether the muscle is behaving like a linear spring which is stretched to a new length as a result of excitation (increase in tension without increase in elasticity) or whether such an imagined spring is not only stretched but also becomes stiffer (increase in elasticity). During the last two and one half years we have been perfecting a method for measuring the viscosity and elasticity of smooth muscles during contraction. This method is described in another abstract in this issue of this journal as well as elsewhere (1950–1952). Results obtained to date indicate that there is a true increase in both the elasticity and the viscosity during isometric contraction produced by an electrical stimulus consisting of pulsating current. The elasticity increases from five to eight times and the increase in viscosity varies inversely though not linearly with the frequency of the applied mechanical sine wave.

Conduction in non-striated muscles. C. LADD PROSSER. No abstract submitted.

AUGUST 12, 1952

The separation and partial identification of the prosthetic groups of cytochrome oxidase and cytochrome b. PHILIP PERSON, W. W. WAINIO AND B. EICHEL.

A method has been developed for the separation and partial identification of the prosthetic groups of cytochrome oxidase and cytochrome b. Na desoxycholate is added in graded amounts to insoluble heart muscle particles. The differential solubility of the components of the cytochrome system permits preparation of two fractions. One contains only cytochrome oxidase and traces of b and c; the other contains only b and c; no oxidase is present. Direct pyridine extraction of the first fraction yields a solution containing the reduced pyridine hemochrome of the cytochrome oxidase prosthetic group. From the second fraction, direct pyridine extraction removes only the prosthetic group of cytochrome b and not that of cytochrome c. The prosthetic group of cytochrome c, under these conditions, is so tightly bound to its protein that it will not complex with pyridine. The above extracts will remain stable for more than a month, without any further treatment, if kept in the cold. The absorption maxima of the reduced pyridine hemochrome of the cytochrome oxidase prosthetic group are at 430 and 587 mμ. Certain preparations show a slight additional absorption at 525–530 mμ. The absorption maxima of the pyridine hemochrome of the prosthetic group of cytochrome b are at 420, 525 and 555 mμ, in the reduced form. The absorption curve of the latter hemochrome is almost identical with that for the reduced pyridine hemochrome of pure ferroprotoporphyrin. Qualitative spectroscopic analysis, and quantitative chemical determination of the metals present in the cytochrome oxidase prosthetic group indicate that Fe is the metal component.

The oxidative pathway of carbohydrate metabolism in E. coli. DWIGHT B. MCNAIR SCOTT.

By the oxidative pathway of carbohydrate metabolism, we mean the oxidation of glucose-6-phosphate to phosphogluconate by the enzyme glucose-6-phosphate dehydrogenase and the coenzyme, TPN, and the oxidative decarboxylation of phosphogluconate to pentosephosphate by phosphogluconate dehydrogenase and TPN. By the use of glucose-1-C^{14} this pathway has been shown to be operative *in vivo* in *E. coli* growing under aerobic conditions to the extent of about 35%.

Several of the enzymes of the pathway have been extracted from the bacteria and separated from each other. Optimal conditions for assay of the activities have been determined. Both enzymes were measured by the rate of reduction of TPN determined at 340 mμ on the Beckman spectrophotometer. When TPN and substrate were present in excess the rate was proportional to the amount of enzyme. Either Mg^{++} or Ca^{++} ions at a concentration of 0.015 or 0.02 M were necessary for optimal activity. The optimal temperature was 36° C. The optimal pH range of the two enzymes differed. Therefore by determining glucose-6-phosphate dehydrogenase at pH 8.5 and phosphogluconate dehydrogenase at pH 7.2, it was possible to determine the separate activities of the enzymes in extracts where both were present.

The enzymes extracted from *E. coli* could account for the oxidation of 4 to 6×10^7 molecules of glucose-6-phosphate and 3×10^7 molecules of phosphogluconate per bacterial cell per minute. During aerobic growth of *E. coli* at 36° C., glucose was used at the rate of 8×10^7 molecules per cell per minute.

Therefore, oxidative enzymes have been extracted to account for 37–80% of the total carbohydrate metabolized.

A comparative study of the ribonucleases of liver and kidney. JAY S. ROTH.

The characteristics of rat liver and kidney ribonucleases (RNases) in homogenates were determined and compared to crystalline pancreatic RNase. The latter is probably an extracellular enzyme with mainly digestive functions while the former are intracellular nucleases whose function is unknown.

Liver RNase had a pH optimum at 6.0 with a second smaller peak at pH 7.8, while the optimum pH of kidney RNase was 7.8 and it also had a second smaller peak at pH 5.9. This may indicate the existence of two enzymes in each tissue. Liver RNase activity in homogenates increased 20–30 per cent after 6–10 days storage at 0°, or after one day in a sample that had been frozen. Under these conditions there was no change in activity in the kidney enzyme. Mg^{++} inhibited the kidney enzyme almost 100 per cent, and the liver enzyme 33 per cent at 0.1 M concentration. The two enzymes responded differently to increasing ionic strength of Na^+ and to heating.

From these and other properties it was concluded that the two enzymes differ from each other and from pancreatic RNase. The kidney enzyme is similar to pancreatic RNase in several respects, but liver RNase is quite different.

Addition of sulfhydryl reactants, p-chloromercuribenzoate, Ag^+, Pb^{++} and methyl glyoxal increased the activity of the liver enzyme in homogenates. Cu^{++}, however, was inhibitory to the extent of 50 per cent at 4×10^{-4} M concentration. The inhibition could be reversed with 2,3-dimercaptopropanol, but this reagent did not affect the reaction rate of the enzyme in control homogenates or those treated with the above mentioned sulfhydryl reactants. Evidence was obtained for the presence of an RNase inhibitor in liver homogenates which was largely or completely absent from kidney.

AUGUST 18, 1952

Cleavage synchrony in individual embryos and in populations of embryos of Arbacia punctulata. ALLAN SCOTT AND HOWARD FOX.

As successive tiers of cells develop within the embryo, a rhythm of division is established. The four vegetal cells of the 8-cell stage cleave from three to five minutes earlier than the four animal cells. The nuclei of the 16-cell stage divide, macromeres first (0 minutes), then mesomeres (+ 5 minutes), then micromeres (+ 10 minutes). The nuclei of the 32-cell stage are arranged in 5 rings, 8 animal-1, 8 animal-2, 8 vegetal-1, 4 vegetal-2 and 4 second micromeres. These rings divide in a progressive sequence: vegetal-1, 0 minutes; animal-2 and animal-1, 5–15 minutes; vegetal-2, 25 minutes; second micromeres undivided. The nuclei of the 64(60)-cell stage divide in a similar sequence (seventh cleavage) beginning with the vegetal-1b ring and progressing towards the animal pole, followed by the vegetal-2 ring of 8 cells, which divides so late as to occur simultaneously with the first divisions of the eighth cleavage, at which time the second micromeres are still undivided; temperature 24° C.

Populations of eggs fertilized simultaneously are well synchronized at the first cleavage but show a progressive loss of coordination in successive cleavages. Embryos fixed at five-minute intervals during the sixth cleavage, stained with Feulgen and mounted whole, show a gradual increase in the average number of nuclei per egg during 30 minutes. At 172 minutes of development 32.9 nuclei per cell, 177 minutes 33.8, 182 minutes 37.0, 187 minutes 39.0, 192 minutes 43.0, 197 minutes 50.8 and at 202 minutes 53.4 nuclei per egg; temperature 20° C.

Conclusions drawn from physiological measurements on individual embryos or on populations must be in accord with the rhythm of division in individual embryos and the progressive loss of synchrony in embryo populations.

The uptake of phosphate during the cleavage of the sand dollar egg. EDWARD CHAMBERS. No abstract submitted.

Oxygen consumption in single marine eggs with special reference to cell cleavage. P. F. SCHOLANDER, C. L. CLAFF AND S. L. SVEINSSON. No abstract submitted.

August 19, 1952

Comparison of dye elimination in the developing liver and kidney (pronephros). Philip B. Armstrong.

When comparisons are made of dye elimination in the developing liver (*Ambystoma maculatum*) and kidney (pronephros of *Fundulus heteroclitus*), particularly as related to morphological and histological differentiation, striking differences are noted in the two organs.

Acid dyes forming true solutions were injected into both *Ambystoma maculatum* and *Fundulus heteroclitus* embryos. Trypan blue was also used.

1. The liver while rudimentary accumulated dyes against a concentration gradient; the pronephros did not.
2. Vascular channels did not appear essential to the elimination of dyes by the liver; elimination took place into the pronephric tubules only after vascular channels developed closely associated with the tubules.
3. Trypan blue was eliminated through the liver after the development of the Kupffer cells; it was not eliminated through the pronephros nor were reticulo-endothelial cells present associated with the pronephros.

Radioiodine uptake in the chick embryo thyroid gland. Seymour H. Wollman and Edgar Zwilling.

Radioiodine uptake by the thyroid gland was studied in twelve 9-day and twenty-three 7-day chick embryos. Forty microcuries of I^{131} were injected into the allantoic vein. After a time interval (0.5 to 4 hrs.) blood samples and thyroids were removed for assay. The carcass below the neck was used as a non-thyroid tissue control. In a few cases samples of fluid and solid yolk, as well as allantoic and amniotic fluids were assayed. Organic binding of I^{131} in some of the thyroids was determined by homogenization in water and protein precipitation. I^{131} was measured by beta or gamma counts.

The I^{131} concentration in the blood fell rapidly after injection and was rarely found to be higher than that expected from a uniform distribution of the dose in the egg. The 9-day thyroids (colloid formation not visible) were found to concentrate I^{131} (ca. 5000 × that of blood); most of this was precipitable. The content of I^{131} in these thyroids was independent of time after injection and of I^{131} in the blood. The 7-day glands concentrated I^{131} but almost none of it was precipitable. I^{131} concentration in thyroids and blood decreased with time after injection, but the I^{131} concentration in the thyroids was 400 to 700 × that of blood at all times. I^{131} in the carcass was ca. 0.7–0.9 × that in blood. The fluid yolk accumulated the greater proportion (55%) of the total dose within a relatively short (1.5 hrs.) time.

These results demonstrate that the chick embryo thyroid gland may concentrate iodide before it acquires its ability to incorporate iodide into organic binding.

The glycogen body as a derivative of the avian neural tube. Ray L. Watterson.

A structural feature peculiar to birds is the glycogen body, a massive glycogen depot between the diverging dorso-lateral funiculi at the level of the four main dorsal roots of the sciatic nerves, separating the two halves of the spinal cord except for the interconnecting ventral white commissure.

Two views of its embryological source have been proposed: (1) an extra-neural origin from pia mater, arachnoid, or both; (2) an intra-neural origin from the wall of the neural tube.

An extra-neural origin after 7½ days of incubation seems ruled out by observation of specimens stained with glycogen-specific stains, since thereafter the reactive cells lie entirely within the roof plate and below the meninges. After 9 days the glycogen body is a single, mid-dorsal structure; between 8⅙ and 7½ days there exist distinct paired primordia separated by a septum of ependymal cells. Fusion of paired primordia progresses dorso-ventrally between 8⅙ and 9 days as this septum narrows and degenerates in dorso-ventral sequence. The youngest stage at which paired primordia can be identified is 7½ days; they are widely separated by a broad ependymal cone and are directly continuous to either side with the adjacent inner mantle of the neural tube wall. Appearances suggest strongly that they represent the dorso-median portions of the inner mantle, *i.e.,* that they are derivatives of the neural tube wall.

Each lateral half of roof plates from three-day neural tubes displaced laterad above skin and feather germs produces a separate glycogen body. Entire roof plates of three-day neural tubes isolated from rest of tube form single glycogen bodies normal in size, shape and glycogen content. Both results favor presence of stem cells within roof plate at three days since relationships of future meninges to roof plate would be highly distorted by operations. Thus an extra-neural origin after three days seems unlikely.

GENERAL SCIENTIFIC MEETINGS

August 26–27, 1952

PAPERS READ

A contribution toward a knowledge of dominance and subordinance in the smooth dogfish, Mustelus canis (Mitchill). W. C. Allee and J. C. Dickinson, Jr.

Dominance-subordinance social patterns having been observed in all classes of living vertebrates except Amphibia, Chondrichthyes and Agnatha, coupled with the fact that such behavior patterns have been well demonstrated in the teleosts, stimulated investigation of this behavior in *Mustelus canis* (Mitchill), the smooth dogfish.

Observation of this species, in groups of 6–10 individuals, revealed certain definite but simple behavior patterns of dominance-subordinance. In 268 noted meetings of two animals, where avoidance was necessary to prevent collision, the smaller individual avoided the larger 251 times. The avoidance motions are smooth and graceful. Aggressive action was observed on only three occasions. No evidence of territoriality was noted.

The behavior pattern of avoidance may be interpreted as an elementary pattern from which more complex types have evolved if it be assumed that this trait is a heritable one in the vertebrate phylum.

It is suggested that an investigation of dominance-subordinance in the Agnatha will prove most interesting and profitable.

The behavior of Spisula eggs with respect to potassium ions. Robert Day Allen.[1]

Isotonic potassium chloride has been employed as a stimulating (parthenogenetic) agent for several marine eggs. In various systems potassium can act either as a stimulant or as an anaesthetic, depending on concentration and other factors. Isotonic potassium chloride will not activate eggs of the surf-clam, *Spisula solidissima;* however, if the potassium ion concentration of sea water is approximately doubled, germinal vesicle breakdown (activation) takes place. As the potassium ion concentration is further raised, increased exposure is required until finally the eggs fail to respond. Activation does not depend primarily upon potassium, for potassium-free sea water is quite as successful as excess potassium in initiating development, and both agents require the presence of calcium ions. When potassium is present in its usual concentration in sea water, Spisula eggs are prevented from developing spontaneously when shed. It is also true, however, that calcium and magnesium ions and other factors also contribute to this natural block to development.

There is a fundamental difference between activation by lack and by excess of potassium; a brief exposure to potassium-free sea water followed by return to sea water will cause activation. However, removal to sea water within five minutes after activation by excess potassium causes reversal of the developmental processes already initiated. This indicates some kind of priming process, the nature of which is unknown.

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[1] Public Health Service Fellow, National Cancer Institute.

The potassium ion concentration also exerts a marked influence on the threshold of ultraviolet light stimulation. Increase or decrease from the normal potassium level in sea water results in a lowering of threshold.

Facilitation of hydrogen ion passage across the chorion of Fundulus by the chlorides of calcium, potassium and sodium. PHILIP B. ARMSTRONG AND D. R. SHANKLIN.

In a series of papers (1915–17, 1922) Loeb described the killing of Fundulus embryos by acetic acid and the delay of this killing by the addition of chlorides of potassium, sodium or calcium; concluding that the presence of the salts antagonized the passage of acid through the chorion. These results were in distinct contrast with those which Loeb obtained with gelatin-coated collodion membranes. The addition of salts to acid increased the rate of penetration of acid through these membranes. He concluded that different mechanisms operated with these two membranes. Armstrong (1928), using de-chorionated embryos of the same age as controls, demonstrated that the major site of Loeb's "antagonism" was at the ectoderm rather than at the chorion. In order to measure more accurately the passage of hydrogen ion across the chorion, the rate of change of pH of the sub-chorionic fluid was determined. The embryos were first washed in distilled water to rid them, insofar as possible, of the sea water salts in the membrane and sub-chorionic fluid, and then in dilute buffer of pH 8.4 to restore the sub-chorionic pH to that of sea water. The embryos were then placed in $M/500$ acetic acid, alone or in combination with salts and the change in sub-chorionic pH was measured by injecting pH indicator dyes into the sub-chorionic fluid. It was found that the addition of salts in most cases increased the rate of passage of hydrogen ions across the membrane and that the relative effectiveness of the chlorides was $Ca > K > Na$. Apparently the chorion of Fundulus is qualitatively the same as Loeb's gelatin coated collodion membrane, the passage of acid across both membranes being facilitated by the addition of salts to the acid. Therefore, the salt-acid antagonism appears to be exclusively at the ectoderm.

Nucleoprotein complexes of sperm nuclei. JAY BARTON II. See abstract in "Reports on Lalor Fellowship Research."

Studies on the poly-D-glutamic acid of Bacillus subtilis NRRL-571.[1] LEON S. CIERESZKO AND CHARLES R. CRANE, JR.

Bacillus subtilis, strain NRRL-571, produces on certain culture media, such as Sauton's, a polypeptide in which the only amino acid present is D-glutamic acid. Poly-D-glutamic acid is produced on simple media containing any one of the following substances: glutamic acid, aspartic acid, alanine, proline, and acetic, pyruvic, citric, aconitic, alpha-ketoglutaric, succinic, fumaric and malic acids. All of these substances are either components of the tricarboxylic acid cycle, or may readily be converted to such. Many of the common organic acids do not serve as sources of the polypeptide. We feel that these results indicate the operation of a tricarboxylic acid cycle in *Bacillus subtilis.*

Poly-D-glutamic acid may be prepared from clarified seven-day cultures of the bacteria on Sauton's medium by the following procedure. The solution is passed through ion-exchange resins to remove ionic material. The peptide stays in solution, and is recovered as the insoluble copper salt by addition of copper sulfate. The copper salt is brought into solution by the use of a complexing agent, such as Versene, and copper ion and added Versene are removed by ion-exchange. The colorless polypeptide solution may be lyophilized to yield a dry product.

Titration of the polypeptide indicates an apparent pH value of 3.9 for its carboxyl groups. The majority of the free carboxyl groups must be gamma carboxyl groups. The polypeptide has a maximum specific rotation in acid solution, and a minimum value in alkaline solution. Sedimentation of the polypeptide preparation in the analytical ultracentrifuge shows it to be homogeneous. The degree of ionization of the polypeptide greatly affects the sedimentation rate. Thus at pH 2.1 the sedimentation rate is approximately 1.5×10^{-13} while at pH 4.1 the sedimentation rate is 1.05×10^{-13}. The sedimentation rate falls further as pH rises.

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[1] Aided in part by a grant from the Frederick G. Cottrell Fund, Research Corporation.

The oxygen consumption of squid nerve. C. M. Connelly. See abstract in "Reports on Lalor Fellowship Research."

Study of the course of formation of the mitotic apparatus in Arbacia and Mactra by isolation techniques. Katsuma Dan,[1] Susumu Ito and Daniel Mazia.

The mitotic apparatus of *Arbacia punctulata* and of *Mactra solidissima* has been isolated successfully by the method described by Mazia and Dan (1952). The following observations have been made. (1) The germinal vesicle nuclei of unfertilized *Mactra* eggs, the nuclei of unfertilized *Arbacia* eggs, and the pronuclei of both types of eggs after fertilization are isolated in morphologically normal form by the method. (2) Monastral figures may be isolated from *Arbacia* eggs shortly after fertilization. (3) In *Arbacia* eggs, distinct spindle fibers are seen to run from the centrioles to the nuclear surface previous to the breakdown of the nuclear membrane. (4) In *Mactra* eggs, we do not observe asters in association with the pronuclei until just at the time when fusion begins. When the pronuclei flatten against each other, two asters are observed. The line connecting their centers runs through the plane of pronuclear contact. (5) In isolated metaphase and early anaphase figures of *Arbacia* eggs, phase-contrast observation distinguishes continuous spindle fibers from pole to pole and fibers corresponding in length and position to chromosomal fibers. The latter had not been observed in *Strongylocentrotus* eggs. (6) In *Arbacia,* the centrioles are frequently seen to have divided at some time during anaphase. (7) In the *Mactra* egg, which cleaves into blastomeres of unequal size, there is a remarkable difference between the two asters. The aster at the pole where the CD cell is to form is much larger than that where the smaller AB cell will form. This relation between size of aster and size of daughter cell in unequal cytokinesis is exactly as predicted by Dan (1943) in his theory of mechanisms of cell division.

The mechanism of nuclear breakdown in the Chaetopterus egg.[2] Lester Goldstein.[3]

Within 7 or 8 minutes after Chaetopterus eggs are shed into sea water the germinal vesicle nucleus breaks down—a necessary prelude to the subsequent maturation divisions of the egg. An attempt has been made to elucidate the physiological mechanisms of this breakdown.

The process evidently occurs in two steps. First, within two minutes after shedding there is a loss of rigidity in the protoplasmic cortex of the egg and this is apparently associated with a release of Ca from the cortex. Once Ca has been released and has performed its function the breakdown process can proceed in the absence of Ca, thus indicating that Ca has activated another factor, probably an enzyme system. (This two-step system may be compared to the two-step system involved in blood clotting, only the first step of which requires Ca.) The nucleus, or the nuclear membrane, is then acted directly upon by the Ca-activated component or system.

It was postulated that the factor activated by Ca might be a proteolytic enzyme. This hypothesis was based on a number of lines of evidence, foremost of which is the fact that crystalline trypsin will produce nuclear breakdown following the removal of Ca from the cell. Moreover, trypsin is believed to enter the cell (rather than to merely function at the surface), since the enzyme apparently acts on the eggs even in media of pH 3.5–4.0. At this pH trypsin is inactive, while the cytoplasm of the egg is not markedly affected. Accordingly, with the foregoing data in mind, extracts of Chaetopterus eggs were made and tested for proteolytic activity by the Anson method, utilizing a hemoglobin substrate at pH 7.5. Preliminary results of these studies indicate that there is present in the eggs a proteolytic enzyme which performs rather much as predicted.

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[1] U. S. Public Health Service Fellow.

[2] This investigation was supported in part by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

[3] Predoctoral fellow of the National Cancer Institute, U. S. Public Health Service.

A study of colloidal changes in sea urchin egg homogenates. PAUL R. GROSS.[1]

Arbacia eggs, freed of jelly, and of their Ca by washings in citrate and Ca-Mg-free artificial sea water, are readily homogenized. When Ca ions are added to such homogenates, a pronounced coagulation reaction takes place, and dense, fibrous aggregates form on the walls of the glass container. This reaction does not occur in the absence of Ca.

The reaction may be followed quantitatively as a decrease in the solubility (in water, 0.5 *M* KCl, and 1 *M* KCl) of the proteins of the homogenate. These solubility changes may be related to the similar changes described by Mirsky for fertilized sea urchin eggs.

The velocity of the clotting reaction depends upon the dilution of the homogenate. The speed falls, and the amount of Ca required to produce a given coagulation in standard time increases markedly, as the homogenate of centrifugally packed eggs is diluted with isotonic KCl. Experiments upon the early stages of the reaction indicate that a brief, transient *increase* in solubility may precede the clotting.

By means of a simple gelatin-digestion method, it was possible to show that the addition of Ca to homogenates induces proteolytic activity which is absent in the Ca-free preparation. The water-soluble proteins of homogenates were precipitated with $(NH_4)_2SO_4$. The precipitate was redissolved and dialyzed. The colloidal material in the sac also became proteolytic in the presence of Ca. This method permitted a determination of the pH optimum of the reaction. The optimum is in the neighborhood of pH 6.6.

These experiments, together with consideration of the inhibitors of the reactions described above, lead to a tentative hypothesis concerning the course of the coagulation reaction. This includes attack by a Ca-activated protease of sensitive protein molecules, which then aggregate.

Electron microscopy of some marine egg surfaces. J. HILLIER, A. I. LANSING AND T. B. ROSENTHAL.

Unfertilized, fertilized, centrifuged and uranyl nitrate-treated eggs of Arbacia, Asterias and Spisula (Mactra) were fixed in buffered osmium, sectioned at approximately $0.2\,\mu$ and studied with an EMU type electron microscope.

The cell surface of the unfertilized Arbacia egg, rather than being smooth is thrown up into small, fairly regularly spaced papillae into which the cytoplasm extends. There is no apparent vitelline membrane. The papillae of the fertilized egg are taller than those of the unfertilized egg. The fertilization membrane is readily recognized as a sheet less than $0.03\,\mu$ thick apparently composed of a single layer of loosely packed particles.

The surface of the unfertilized Asterias egg is quite similar to that of Arbacia. The papillae are somewhat broader and a vitelline membrane closely adherent to the surface may be distinguished.

The surface of the Spisula egg is quite distinctive. It is roughly $1\,\mu$ thick and possesses three characteristic layers through which long papillae or pseudopods course. The papillae arise from the cell surface as single stalks which branch into an average of four in the second layer. The three layers are of graded density with the outermost being most dense. Sections perpendicular to the long axis of the papillae show that they have a regular hexagonal-net arrangement in the outer layer, a less regular but similar arrangement in the middle layer and, in the inner layer where they join into the stalks, no distinguishable pattern.

Effect of temperature on the birefringence of the mitotic spindle. SHINYA INOUÉ. See abstract in "Reports on Lalor Fellowship Research."

The frequency dependence of the mechanical properties of smooth muscles. WILLIAM H. JOHNSON, JOHN F. PERKINS, JR. AND ARNOLD M. KATZ.

The elasticity and the viscosity (mechanical resistance) of the anterior byssal retractor muscle of the mussel, *Mytilus edulis,* were measured by a method described briefly as follows:

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[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

A sinusoidal stretch is applied to the muscle and the sinusoidal changes in tension passively developed by the muscle as the result of the stretch are measured. From the knowledge of the amplitude of this tension, the amplitude of the sinusoidal stretch and the phase angle between the two sine waves, the elasticity and the mechanical resistance (viscosity) can be calculated in a manner analogous to the measurement of capacitance and resistance using the impedance bridge. These quantities, the viscosity and elasticity, were measured at frequencies of sinusoidal stretch ranging from 0.1 c.p.s. to 6.7 c.p.s. both during rest and during isometric contraction. In recent experiments the physiological response and the length at which tension first appeared upon stretching the muscle remained constant in most cases. The results obtained further substantiate earlier findings that the viscosity is frequency-dependent whereas the elasticity is virtually independent of frequency. The viscosity increases with decreasing frequency to a greater extent during contraction than during rest. This suggests greater cross-bonding during contraction (following Eyring and Tobolsky).

Electron microscopy of some marine egg inclusions. A. I. Lansing, J. Hillier and T. B. Rosenthal.

Ultra-thin sections of buffered osmium fixed eggs of Arbacia, Asterias and Spisula (Mactra) were studied with the electron microscope.

The cortical granules of Mactra appear to be homogeneous spheres while the granules of the other two eggs studied seem to be composed of spheres of a matrix material with peripherally embedded inclusions. Upon fertilization in Mactra there is an increase in the number of cortical granules at the surface followed by a sharp decrease in their density. Upon fertilization the cortical granules of Arbacia rupture, discharging the contents of the peripheral inclusions, and leaving the remaining vacuole-like structure as an extension of the cell surface.

Unfertilized Arbacia eggs centrifuged at high speed (75,000 rpm for 20 seconds) contain two layers of what appear to be mitochondria, one in the centripetal lipid layer and the other just above the yolk granule layer. The clear middle zone contains numerous elements described by McCulloch as the coarse fibrous component. However, serial sections show that these are sheets rather than fibers and are composed of double membraned lens-like bodies separated by cross striations.

As with light microscopy, the resting nucleus contains little formed structure but during division, very dense chromosomes may be seen.

Desoxyribonucleic acid in Arbacia eggs determined by isotope dilution. A. Marshak and C. Marshak.

Methods previously used for DNA determination in this and related materials can be shown to be inadequate. The isotope dilution method with C^{14}-thymine is free of these errors. C^{14}-thymine was isolated from *Thiobacillus thioparus* grown in a medium where the only carbon source was $C^{14}O_2$ and purified by column and paper chromatography. The final product was at least 98% pure by radioactive and spectrophotometric criteria. Eggs were treated to obtain a soluble fraction containing RNA nucleotides and an insoluble residue containing DNA. No thymine was found in the former and no uracil in the latter, indicating adequate separation of DNA and RNA. On the basis of uracil determinations there was found to be 24×10^{-4} μg RNA/egg. The DNA fraction was digested with $HClO_4$ and C^{14}-thymine added immediately afterward. After equilibration, thymine was isolated from the digest by paper chromatography and purified by four successive chromatographic procedures. The plating and counting procedures were found by preliminary trial with C^{14}-glycine to be reproducible within an error of 1%. From the specific activities of the added and recovered thymine it was calculated that there was 8.4×10^{-7} μg thymine or 8.1×10^{-6} μg DNA per egg. Direct analysis by paper chromatography gave 8.2×10^{-8} μg thymine or 7.9×10^{-7} μg DNA per sperm. As demonstrated by cytological procedures, this result must not be interpreted as meaning that there is 10 times as much DNA in the egg nucleus as in the sperm.

Desoxyribonucleic acid in the nuclei of Arbacia eggs. A. MARSHAK AND C. MARSHAK.

Suspensions of extruded eggs washed and extracted as previously described were stained by the Feulgen reaction. Live preparations were examined by phase contrast. In addition to ripe ova, some suspensions contained oocytes and small fragments of the ovary. Oogonia and oocytes were enveloped by numerous syncitial or nurse cells with strongly Feulgen-positive nuclei, some of which appeared to be embedded in the egg cytoplasm and were frequently pycnotic. The nuclei of young oocytes have chromatin as typical paired strands that are very delicate but are definitely stained red. On enlargement of the nucleus the capacity to stain is lost. Chromosomes of the polar body divisions are bright red. However, there is no color in the pronucleus of the mature egg even though the chromatin strands are coarser than those of the early meiotic stages which do stain. The size of the pronucleus is not significantly different from that of nuclei through the 16-cell embryo stage. There is a progressive increase in intensity of staining of resting nuclei of successive cleavage stages. The evidence indicates a loss of DNA by nuclei in the early meiotic stages, and its absence in the pronucleus. The capacity of the pronucleus for producing DNA is indicated in the polar body divisions. The large amount of DNA found by chemical analysis of egg suspensions cannot be assigned to either the nuclei or the cytoplasm of the egg unless the presence of nurse cells is eliminated. Since Arbacia may develop parthenogenetically, it follows that DNA must be a derived product and not a prime originator of the reactions occurring at specific chromosome loci that are considered to be essential gene functions.

The osmotic behavior and anatomy of the Fundulus chorion. D. R. SHANKLIN AND PHILIP B. ARMSTRONG.

In 1905 Loeb stated that Fundulus choria were impermeable to water, later modifying this to "practical impermeability" under physiologic conditions and in distilled water. In 1908 he explained the collapse and crenation of the eggs in sucrose solution by exosmosis of salts. Yagle (1930) discussed this problem of salt and water passage, but failed to demonstrate endosmosis of water. By this study, the permeability of choria to water in both directions was measured. Exosmosis was measured by a manometric device, and passage of water both ways was measured by a geometric consideration of the collapse of the chorion in sucrose solution, and its recovery in distilled water or sea water. A mathematical treatment voids the salt exosmosis explanation for this behavior. Sucrose itself will pass this membrane, for a collapsed shell left in sucrose will recover, thence the yolk sac crenates and the embryo dies. These choria are resilient and spring back into shape with much force and speed. It is this quality that yields a discrepancy in the permeability of the chorion to water, for the water passage of recovery is three times that of collapse. If the chorion had a number of pores of sufficient size, this resilient "sieve" would "move through the water," and "capture" water to restore its spheroid shape. Microscopic examination reveals: (1) a chorion externum; 7–8 μ thick; with 10–20,000 pores/mm.^{2}, of 1–1.5 μ diameter; cylindrical with flanging at the outer opening, circular to polygonal in profile; randomly distributed; (2) a chorion internum; 3–4 μ thick; composed of protein fibrils 1–1.4 μ diameter, arranged in a herringbone manner, as a meshwork. Electron microscopic examination reveals no finer structure to the external chorion, thus a homogeneous layer of matrix, and the evidence that some of the pores pass both layers. The internal chorion is the site of osmotic regulation, while the outer affords mechanical protection.

Glycolytic activity in the swim bladder gland. C. F. STRITTMATTER, E. G. BALL AND O. COOPER. See abstract in "Reports on Lalor Fellowship Research."

The relation between the excystation of an apostome ciliate (Gymnodinioides sp.) and the molting of its crustacean host (Uca pugnax). WILLIAM TRAGER.

Small oval cysts of a species of *Gymnodinioides* have been found on the gills of over 80% of the fiddler crabs examined. Excystation of the ciliates has been observed only in material taken from crabs during the act of molting or just before molting. The observations show that excystation begins just before the crab begins to molt, and that the excysted forms make their way into the shed cuticle of the legs during the act of molting. When fragments of infected gill taken from crabs during molting were kept in a mixture of sea water and blood of the crab, fully engorged ciliates (trophonts) developed within about 9 hours. Encystation of the trophonts and the development of small daughter ciliates required a further two days. Cysts on the gills of crabs not near molting underwent no change when kept for one or two days in the milky-white blood of crabs about to molt.

PAPERS READ BY TITLE

On the presence of lactose in the hypobranchial gland of the snail, Busycon canaliculatum (L.). Metry Bacila and R. R. Ronkin.[1]

This note reports the occurrence of lactose, possibly as lactosamine, in a marine mollusk. Lactose is commonly considered a biochemical attribute of mammals.

Fresh mucus was obtained by agitating the excised hypobranchial gland of *Busycon* in a small volume of distilled or sea water. The secreted mucus was separated from the gland, precipitated with two volumes of 95% ethanol and allowed to stand three hours at 5° C. The precipitate was separated by centrifuging and decanting, washed by centrifuging three times with 95% ethanol, once with 100% ethanol and once with ether, and dried. The dried material, redissolved in water, gave positive biuret (protein) and Molisch (carbohydrate) tests, but after precipitation with $Ba(OH)_2$ and $ZnSO_4$ the supernatant was negative to both tests.

The dried, alcoholic precipitate was hydrolyzed by refluxing 50 mg. with 5 ml. 0.12 N HCl for four hours, cooled and centrifuged. The supernatant was neutralized to pH 7 with $NaOCH_3$ or NaOH and centrifuged. The clear supernatant contained free reducing sugar (Tollens's ammoniacal $AgNO_3$, Somogyi's and Nelson's reagents). Treatment with phenylhydrazine gave the characteristic crystals of lactosazone only, or, with more intensive hydrolysis, lactosazone, glucosazone and galactosazone. Paper chromatograms (solvent, BuOH-HAc; developer, Partridge's aniline-phthalate or benzidine) gave the same results, with the possibility that the lactose may have been in the form of lactosamine.

The polysaccharide was separated from fresh mucus by stirring 90 minutes with $CHCl_3$, centrifuged and the aqueous phase, containing no reducing sugar, precipitated with ethanol. The washed precipitate was hydrolyzed (0.5 N H_2SO_4, 100° C., four hours), neutralized to pH 4.5 with $Ba(OH)_2$ and centrifuged. The supernatant, concentrated by evaporation and chromatographed (solvent, phenol or BuOH-HAc with NH_3 and KCN added to each; developer, acetyl acetone and *p*-dimethylaminobenzaldehyde). The results suggested the presence of amino sugar and free hexose. It is possible that the amino sugar is glucosamine and that the hexose is galactose. Lactose, possibly as lactosamine, is thus the principal, if not the sole hydrolytic product of the polysaccharide of this mucus.

An ecological study of coastal plants on Cape Cod. Steve G. Boyce.

Salt spray, droplets of sea water concentrated by evaporation and transported inland by coastal winds, has been shown to be a factor in the ecology of plants along the shores of Cape Cod. The highest intensities of salt occur with the prevailing southwesterly winds and with storm winds of the northeast; consequently the espalier shape of coastal plants is oriented in these respective directions. The difference in species and in physiognomy on the southwest and on the northeast coast is due to the difference in frequency and fetch of the winds on the respective coasts. Increasing length of fetch increases salt spray intensity due to increased wave activity, increased swash and consequently increased effervescence of droplets. Anatomical studies of leaves of plants exposed to different intensities of salt spray showed that chloride-induced hypertrophy was present in all species (32) except grasses. The degree of hypertrophy attained before coagulation of the protoplasm was correlated with the degree of tolerance of the species to chloride salts.

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[1] Aided by a grant from the Research Committee of the Faculty of the University of Delaware, and by a contract between the Office of Naval Research, Department of the Navy, and the University of Delaware (160-015).

Modification of the tidal rhythm of Uca by tidal difference and by illumination. FRANK A. BROWN, JR., MURIEL I. SANDEEN AND MILTON FINGERMAN.

The integumentary melanophores of the fiddler crab, *Uca pugnax*, exhibit both a diurnal and a tidal rhythm. To determine the influence of time of low tide at two separate locales upon the phases of the tidal rhythm, *Uca* were collected at Chapoquoit and Lagoon Pond and their tidal rhythms under constant laboratory conditions compared. The time of occurrence of the maxima and minima of the tidal rhythm of the Chapoquoit *Uca* averaged 4.3 hours earlier than for the Lagoon Pond animals. The actual tidal difference between these two regions is 4 hours. It is, therefore, evident that the phases of the tidal rhythm of *Uca* are determined by the time of low tide in the environment. To demonstrate whether the tidal and diurnal rhythms of *Uca* are independent or associated with one another in some fashion, *Uca* were collected at Chapoquoit and divided into two groups. Both lots were placed in darkness at 9 P.M. on the day of collection. One lot was illuminated from midnight until 6 A.M. for three nights to shift the diurnal rhythm backward approximately a quarter cycle; the other lot serving as a control. A comparison of the times of maximum and minimum augmentation of the degree of dispersion of the melanophores indicated that the diurnal rhythm had actually been shifted backward five hours and the tidal rhythm 4.2 hours. The difference between five and 4.2 was not considered significant because of the difficulty of determining exactly the times of the maxima and minima of the tidal rhythm. It is evident, therefore, that the tidal rhythm is in some manner superimposed upon and associated with the diurnal rhythm. The tidal rhythm is probably adaptive for each environment since *Uca* habitually feeds at low tide.

A persistent tidal rhythm in the fiddler crab, Uca pugnax. FRANK A. BROWN, JR., H. MARGUERITE WEBB AND ROBERT C. GRAVES.

It is well known that the black pigment of *Uca pugnax* exhibits a diurnal rhythm of dispersion during the day and concentration at night and that this rhythm persists for several weeks in constant darkness. The state of the melanophores of a group of *Uca pugnax*, maintained in a photographic darkroom, was determined at hourly intervals from 5 A.M. to 10 P.M. inclusive, each day during a 26-day period. When these states are plotted against time in hours it is seen that the form of the daily excursion varies considerably from day to day. The curve may be almost perfectly symmetrical with maximal dispersion near noon; it may be skewed with maximal dispersion in either morning or afternoon, or it may be bimodal with a decreased dispersion at about noon. These periods of maximal dispersion were seen to bear a definite temporal relationship with low tides at the place the animals were collected. Further analysis of this relationship was performed by summing the chromatophore values for a given day and then determining the per cent of the total value represented by each of the six three-hour periods. The percentage values for any given three-hour period, when plotted against time in days, show a clearly rhythmical variation throughout the experimental period. The cycle so obtained has a frequency which approximates the 14.765 day half-lunar cycle of the tides. The minima (and maxima) progress from one 3-hour period to the next at the same rate as the tides. The observations indicate that the daytime dispersion of *Uca* black chromatophores is amplified in association with the time of low tide, that the precise time-relations between maximal dispersion and occurrence of low tide vary systematically throughout the day, and that this tidal rhythm persists for at least 26 days after the animals have been removed to a constant environment.

On the relationship between cleavage block by respiratory inhibitors and viscosity changes in Ilyanassa eggs. JOSEPH M. BUTROS.

To investigate the possibility that a change in the physical state of the cytoplasm (viscosity) links the chemical (oxidative) inhibition with the biological (mitotic) block, the following inhibitory agents and anesthetics were used: $M/200$ sodium azide at pH 6.4; $M/200$ iodoacetic acid at pH 6.2; $M/1000$ KCN; 0.003% trichlorophenol; 0.01% dinitrophenol; 0.0018% maleic acid at pH 7; 1.5% thiourea; 0.06% phenylurethane; and 0.15% chloral hydrate. Viscosity of the eggs was studied by the centrifuge method; surface rigidity by the amount of elongation after centrifuging also. Some agents increased the viscosity, others decreased it; and still others did not affect it. In lower concentrations of some agents (azide) the delayed eggs cleaved in spite of the changed viscosity. Thiourea and chloral hydrate decrease the viscosity, maleic acid has a weak increasing effect. In azide, eggs became flattened, failed to cleave and became very viscous. When returned to sea water, they rounded up and after the return of viscosity to normal, they divided. Iodoacetic acid effect is like azide in general but irreversible after 20 minutes. Cyanide does not block cleavage, nor change viscosity, but causes the cortex to become more rigid. The substituted phenols increase the viscosity and make the cortex more rigid. In urethane the cortex is less rigid but the viscosity is increased.

From the above data, action of the agents on viscosity through cytochrome oxidase is eliminated. Other enzymes like phosphorylases could be involved. It is possible that these agents act directly on the surface and cytoplasm, changing viscosity and rigidity, respectively. It can at least be said that whenever a metabolic inhibitor blocked cleavage, it produced a change in viscosity and usually a change in surface rigidity.

Chemical characteristics of rotenone antagonists as determined by effects on dividing sea urchin eggs.[1] IVOR CORNMAN AND EDWARD F. ROGERS.

Using dividing sea urchin eggs, it was previously shown that vitamin E phosphate completely reversed a cytolysing dose of rotenone (0.008 mg./L) and that vitamin K_1 reversed weaker doses (0.004 mg./L). Related substances are being tested to discover the chemical characteristics necessary for rotenone antagonism. Vitamin K_1 oxide is partially effective against rotenone, but menadione (a mitotic poison itself, as shown by Heilbrunn and Wilson) and menadione phosphate are ineffective despite their vitamin K activity. Vitamin A has a carbon chain similar to that in K_1 and E. A-acetate and A-palmitate both partially reverse rotenone. Phytol alone is weakly effective in emulsions 10 times as dense as effective concentrations of the vitamins. It therefore appears that diverse ring structures (benzopyran, naphthoquinone, or partially saturated carbon ring) can improve the antagonistic action of the carbon chain even though a ring system alone (menadione phosphate) is ineffective.

In the range of 10 mg./L the vitamins in oil form neutralize the effect of rotenone to the amount of 0.001 mg./L. E phosphate, the most soluble form, requires 40 mg./L to neutralize 0.001 mg./L of rotenone. The fact that reversal of rotenone poisoning has been obtained only in the presence of precipitates or emulsions enjoins consideration of a purely physical action involving adsorption or solution, even though purely solvent action is inadequate to explain the difference between antagonistic and inactive oils.

On the other hand, the possibility of competition between vitamin E and rotenone at some site in or on the egg is strengthened by complete recovery of eggs blocked for an hour by rotenone (0.008 mg./L) before E phosphate is added. Washing such rotenone-poisoned eggs yields only abnormal blastulae.

Effects of P^{32} on mitosis in Chaetopterus eggs.[2] DONALD P. COSTELLO, CATHERINE HENLEY AND DONALD E. KENT.

Chaetopterus eggs were treated, beginning two minutes after insemination, by placing them in 90 ml. sea water containing varying amounts of P^{32}. The duration of exposure ranged from 40 minutes to 9 hours, and the concentration of P^{32} from zero to 0.07 millicuries per milliliter. The calculated total decay in the dishes containing the eggs ranged from zero to 100,000 R. E. P., though only a fraction of this dosage could reach the eggs. Egg samples were removed at intervals from the treated and control cultures, fixed as flattened whole mounts, and prepared for cytological examination by the Feulgen technique.

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[1] Aided in part by an American Cancer Society institutional grant to the George Washington University Cancer Clinic.

[2] Work done under A. E. C. Contract AT-(40-1)-1085.

When a concentration of approximately 0.03 mc./ml. is reached, the early mitotic divisions are markedly affected. At this dosage, for eggs fixed after 40 minutes exposure, very large polar bodies, multiple asters in the cytoplasm and chromosomes of abnormal appearance are characteristic effects. Higher dosages (up to concentrations where all mitotic activity is stopped) result in chromosomal fragmentation and some multipolar spindles. Embryos fixed after three to five hours exposure show similar effects in the 8- to 16-cell stage cleavage blastomeres, plus some spectacular multipolar and polyploid figures. These latter often show large masses of chromosomes and apparent suppression of cytoplasmic divisions, reducing the cell number considerably below that of the controls. At the highest dosages used, both maturation divisions and cleavage were completely suppressed.

The fertilization inhibitor in the blood cells of Arbacia.[1] PIERRE COUILLARD.

In work frequently quoted, Lillie (1914) and later Lillie and Just (1924) stated that the perivisceral fluid of Arbacia inhibits fertilization. Oshima (1921) and more recently Pequegnat (1948) showed that the fluid itself contains no inhibitor and that Lillie's results were due to contamination by a "yellow substance" diffusing from pigmented haemocytes in the dermis during a preliminary washing of the animals with tap water.

As a matter of fact aged blood does inhibit fertilization. This is due to a substance, apparently echinochrome, which escapes from the pigmented blood cells.

Also Lillie's results might be explained by his use of filtered blood, for certain types of filter paper, in contact with blood or sea-water, yield a fertilization inhibitor.

Crystalline echinochrome was prepared by extracting blood cells and was found to inhibit fertilization. The effect is reversible; inhibited eggs fertilize readily if washed. If eggs are exposed after fertilization, echinochrome has no effect on cleavage. However, when fertilization is attempted in low concentrations of echinochrome, the eggs which are fertilized show on cleaving a separation of blastomeres such as occurs in calcium free sea-water. (A similar observation was made by Pequegnat.)

The binding of calcium by echinochrome was then investigated. Chemically, one mol of this compound could combine with 2½ mols of calcium (Tyler, 1939) and the complex formed precipitates in concentrated solutions. Addition of a calcium salt or of sea-water to echinochrome in distilled water modifies radically the absorption spectrum. Biologically the effects of echinochrome on fertilization and cleavage can be duplicated by solutions of potassium oxalate and prevented by excess calcium.

Mixed with sperm suspensions, dilute echinochrome causes a prolonged perhaps irreversible agglutination; in higher concentrations, sperm soon loses its motility, due presumably to a lack of free calcium (cf. Tyler, 1939).

It is suggested, therefore, that echinochrome acts primarily as a calcium binder in preventing fertilization.

The role of the midpiece in the incorporation of P^{32} into the ribonucleic acid of Arbacia spermatozoa. HENRY DI STEFANO AND DANIEL MAZIA.

Living Arbacia spermatozoa in sea water containing $P^{32}O_4$ (1 microcurie per ml.) incorporate P^{32} into the organic fractions insoluble in cold 10% trichloroacetic acid. The total activity incorporated into nucleic acids was estimated by measurements of the radioactivity of the fraction soluble in hot 5% TCA. In parallel samples, the partition of the P^{32} between DNA and RNA was determined by measurement of the activity released by crystalline desoxyribonuclease and ribonuclease, respectively. No detectable amount of P^{32} was incorporated into DNA, which is in agreement with observations on other types of cells. On the other hand, the amount of P^{32} liberated by ribonuclease was identical with that extracted with hot TCA.

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[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

Therefore the sperm cell, in spite of its very small amount of cytoplasm and its lack of capacity for growth, does turn over or synthesize RNA.

A technique has been developed for isolating the midpieces of sea urchin spermatozoa in quantity. This involves, essentially, removal of tails by centrifugation in citrate solutions and heads by lysis in 2 *M* NaCl. When midpieces were isolated from samples of Arbacia spermatozoa that had been incubated in P^{32}, about 30% of the hot TCA-soluble P^{32} was recovered with the purified midpiece preparation. Since the method at present does not guarantee 100% recovery of midpieces, it is concluded that these particles are an important and perhaps the major center of RNA activity in the sea urchin sperm cell.

Comparison of the action of sodium adipate and sodium malonate on succinic dehydrogenase activity. Bertram Eichel and W. W. Wainio.

Succinic dehydrogenase was assayed in rabbit liver homogenates spectrophotometrically. A concentration series of 8.3×10^{-5} *M* to 3.3×10^{-3} *M* sodium malonate in the presence of a fixed concentration of sodium succinate (3.3×10^{-3} *M*) gave 64 to 92% inhibition, while a concentration series of 3.3×10^{-3} *M* to 2.3×10^{-2} *M* sodium adipate gave 0 to 70% inhibition. A plot of 1/v vs. inhibitor concentration for these data (J. M. Reiner—private communication) indicated that normal inhibitor kinetics are applicable to this situation. The data also demonstrated that the amount of inhibitor-enzyme complex formed in each case as compared to the total concentration of inhibitor is not negligible.

It has been known that malonate behaves as a competitive inhibitor towards succinic dehydrogenase. The type of inhibition given by adipate, however, has thus far not been demonstrated.

With a fixed inhibitor concentration of adipate, 1.98×10^{-2} *M*, and malonate, 8.3×10^{-4} *M*, a concentration series of 8.3×10^{-5} *M* to 6.6×10^{-3} *M* succinate was studied in the presence of enzyme. Analysis of the malonate data by the method of Lineweaver and Burk, and Reiner, demonstrated that malonate does behave as a normal competitive inhibitor towards succinic dehydrogenase for all concentrations of succinate assayed. The Lineweaver and Burk analysis of the adipate data indicated possible competitive inhibition of the enzyme for a concentration range of succinate from 8.3×10^{-5} *M* to 1.65×10^{-3} *M*. Analysis by the method of Reiner ruled out the possibility of pure non-competitive inhibition and demonstrated that adipate behaves either as a pure competitive type or a mixed type of inhibitor. If adipate is a mixed type, its ability to combine with the free enzyme (competitive) far exceeds that of its combination with the enzyme-substrate complex (non-competitive).

Respiration and enzyme studies on X-irradiated Tetrahymena geleii W. Herbert J. Eichel and Jay S. Roth.

Recently, a study of the effects of total body x-irradiation on a number of rat tissue enzymes was completed (in press). It was believed that a similar investigation on unicellular animals might be of some aid in explaining the high radio-resistance of these organisms. The effect of x-irradiation on the activities of ribonuclease and desoxyribonuclease and on respiration under various conditions was studied. In each experiment standardized 48-hour cultures of Tetrahymena were concentrated by centrifugation, washed with distilled water and diluted to a constant volume. In respiration studies samples were removed for controls or to be irradiated and were then added to Warburg flasks containing 1.0 ml. of 0.1 *M* phosphate buffer, pH 7.4. Ribonuclease and desoxyribonuclease were assayed by the turbidometric method of McCarty on homogenates prepared from whole cell concentrates. Dry weight determinations were made in triplicate at 80° on the whole cells or homogenates. The activities of the nucleases were not changed significantly in homogenates obtained from cells which were irradiated at 300,000 and 500,000 r. This is to be compared with wide fluctuations in activity observed previously in the case of rat kidney and liver ribonucleases in animals, exposed to 500 or 750 r. When the oxygen consumption of Tetrahymena exposed to 300,000 r was measured immediately after irradiation, the QO_2 of the irradiated organisms decreased about 30%. After 24 hours, however, the respiration of the x-irradiated cells increased significantly above the controls.

Irradiation of the cells in the presence of 8.3×10^{-3} *M* L-cysteine and DL-homocysteine afforded approximately 45% protection. L-methionine gave no protection. It was noted that L-cysteine (final concentration 3×10^{-3} *M*) stimulated the QO_2 of normal cells by 34% while equimolar quantities of glycine, L-alanine, L-methionine, DL-homocysteine and glutathione had no effect on endogenous respiration.

The occurrence of mating types in Tetrahymena. ALFRED M. ELLIOTT AND DAVID F. GRUCHY.

One hundred twenty-seven clones of *Tetrahymena* sp. (probably *pyriformis*) were established from 15 different fresh water habitats in the Woods Hole area. Of these, three mated with themselves (selfers), whereas all others failed to conjugate within the clone (non-selfers). By making numerous combinations of non-selfers, both from the same habitat and from different sources, two mating types were finally discovered. These came from the same pond and mated readily with all clones from that source except one. Conjugation was obtained in only two of the remaining 101 clones.

Employing antibiotics, the two mating types were established in axenic cultures. They grow abundantly in proteose-peptone or the synthetic medium which is satisfactory for *T. pyriformis* strain E. Their nutritional requirements examined so far resemble closely those of strain E.

The procedure employed routinely in obtaining conjugation is as follows: Organisms (bacterized or axenic) are washed once with distilled water by centrifugation. They are then mixed in depression slides in distilled water, after which the first pairs appear in approximately 4 hours. If the original mating types are mixed in equal numbers approximately 100% of the cells will conjugate within 12 hours. The complete process requires about 8 hours, after which they separate and begin dividing, following a 5–6 hour period of feeding. They conjugate readily at room temperatures (18–26° C.) and at a pH above 7.0.

Improved culture conditions for Tetrahymena. A. M. ELLIOTT, J. F. HOGG, J. V. SLATER AND C. WU.

Studies were made on the physical and chemical factors involved in the growth of *Tetrahymena pyriformis* E. Of the physical factors, temperature and the effect of surface area were studied. Two temperature peaks were found for maximal growth rate (24° and 28° C.). The best growth was obtained when the ratio of surface area to volume was highest.

Of the chemical factors, studies were made (1) to discover whether the amino acid composition could be modified and lowered in amount with a view toward supplying available nitrogen from other sources, and (2) to determine the necessity for the addition of certain cations to the basal medium. When all l-amino acids were in the same proportion as in the medium routinely used, it was found that each could be reduced by one-half, with some reduction in growth rate. When amino acid levels were adjusted to twice the base level, on the basis of direct utilization data, and asparagine added to increase the amount of nitrogen, better growth was obtained than in the controls. Non-essential amino acid mixtures increased the growth rate and maximal growth when added to media constructed on the basis of minimal growth requirement data. The addition of asparagine to six times this base level resulted in the best growth rates and the highest maximal growth. This spectrum is recommended as the most economical for general use.

Magnesium and potassium are essential for strain E. This ciliate also probably requires less than one microgram/ml. each of copper and iron. Best growth is obtained when salts of magnesium, potassium, copper, iron and zinc are added to the synthetic environment. Without extensive purification procedures, however, it is impossible to demonstrate definitive cation requirements for this animal.

The effects of hematoporphyrin and other substances upon x-ray sensitivity of Paramecium. FRANK H. J. FIGGE AND RALPH WICHTERMAN.[1]

Various substances have been found to alter radiation sensitivity in vertebrates. In such multicellular forms, it is impossible to determine whether the effect is one which involves direct action of radiation on cells or an indirect effect due to complex secondary reactions and interactions of groups of highly specialized cells. For instance, stress reactions augment the influence of total body radiation in mammals. The protective effect of sodium pentobarbital in irradiated mice is perhaps related to the inhibition of stress reactions. Paramecia are ideal for the analysis of some of these radiation problems.

Using methods and conditions described elsewhere in these pages, specimens of *Paramecium caudatum* were irradiated in control media and in media containing hematoporphyrin, sodium nitrite or sodium pentobarbital. Paramecia in control media require very high dosages of irradiation to produce lethal effects (LD 50, 24 hr., 350 kr). Paramecia irradiated in chemically non-toxic porphyrin concentrations of 1 : 10,000 to 1 : 40,000 caused 100 per cent mortality with 100 kr within 12 hours. It is known that hematoporphyrin sensitizes paramecia to visible and near ultraviolet light. The above and other experiments demonstrate in a conclusive way that porphyrin sensitizes paramecia to short wave-length radiations such as x-rays.

Preliminary experiments with sodium pentobarbital and sodium nitrite in the concentrations employed (1 : 1000–1 : 4000) indicate that these substances do not protect paramecia from lethal effects of x-radiation. The protective effect of sodium pentobarbital in mammals is, therefore, possibly related to its tendency to suppress stress reactions which involve the nervous and endocrine systems.

The passage of vital stain from host to ectoparasitic wasp (Habrobracon). DANIEL S. GROSCH.

The present note reports a feasible means of experimental introduction of chemical agents into Habrobracon larvae. An intended application is to the investigation of the effects of ingested radioactive isotopes (A.E.C. Contract No. AT-(40-1)-1314). In order to have visible evidence of the distribution of substance introduced, 1% solutions of a chemically representative series of stains, most of them termed "vital" by stain technologists, were considered in these explorations. Paralyzed (pre-stung) caterpillars of the normal host, Ephestia, were given posterior injections using needles fashioned from glass capillary tubing. Habrobracon eggs were placed on the injected hosts and incubated at 30° C. Daily observations were made of the parasites which developed from the eggs.

With the notable exception of neutral red and toluidine blue, coloration was restricted to the midgut of the parasite and egested with the meconium. When these two stains were employed the appropriate color appeared in the urate cells. This phenomenon developed particular prominence with neutral red where the color persisted into the adult. Furthermore, pink cocoons were spun by the tinted parasites. Non-toxic stains restricted to the gut contents included fast green, alizarin red S, vital red, trypan blue and cresyl blue. Relatively toxic stains which caused premature deterioration of the host and death of the parasite include safranin O, Janus green B, trypan red and hematoxylin. Experiments with methylene blue were unsatisfactory because of extensive decolorization by the host. Only about 3% of the larvae fed on colored areas and showed slight coloration of urate cells. Although those stains which reached the urate cells belong to the quinone-imine group, it should be noted that cresyl blue and safranin O are also included therein. Therefore the structural chemistry of the chromophore groups cannot be advanced in explanation of absorption of a stain. Perhaps relatively low molecular weight and simplicity of the dye molecule as a whole may be significant.

Depolarization and contraction in smooth muscle on rapid cooling. RITA GUTTMAN, ARNOLD M. KATZ AND JOHN F. PERKINS, JR.

The anterior byssal retractor muscle of *Mytilus edulis,* the fibers of which are parallel and run the full length of the muscle, was rapidly cooled from about 24° to 7° C. Resting potentials were recorded on a Grass ink-writing apparatus through a cross-coupled input D. C. amplifier, A. C. being eliminated by means of a Wagner ground. A kymograph was used to record virtually isometric contractions. The muscle was placed in a lucite chamber. One end of the muscle was maintained in 0.48 M KCl and the other end was subjected to experimental solutions with a paraffin oil compartment between. Rapid cooling of the muscle in sea water caused reversible partial depolarization, but no contraction. Room temperature solutions containing three to five times the amount of KCl normal in sea water resulted in much slower partial depolarization and very slight contraction. Upon rapid cooling, these high potassium solutions potentiated a contraction accompanied by increased depolarization. Thus, the effects of cold and high potassium may be additive. Adding saturated cocaine in sea water to the 0.48 M KCl compartment resulted in no change of potential. Adding it then to the experimental compartment provided a true zero potential for the preparation. It is hoped that these experiments may contribute to the understanding of the relation between depolarization and contraction in muscle.

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[1] This work was supported by the Committee on Research, Temple University, the Office of Naval Research, Department of the Navy and the American Cancer Society (Maryland Division).

A further study of the mitotic inhibitor in starfish extracts.[1] L. V. HEILBRUNN, A. B. CHAET, ARNOLD DUNN AND W. L. WILSON.

Heilbrunn, Wilson and Harding (1951) showed that acid sea water extracts of starfish ovary have a marked liquefying and anti-mitotic action on Chaetopterus and Arbacia eggs. Such extracts also prevent cleavage of eggs of the clam Spisula and they prevent germinal vesicle breakdown and polar body formation in the starfish egg.

The potency of the extract is completely destroyed by periodate. This indicates that the effect may be due to a carbohydrate. If a carbohydrate is responsible, it appears to be combined with a protein. Autoclaving destroys the activity of the extract. When the crude extract is salted out with varying concentrations of ammonium sulfate, the potency remains in the globulin fraction. If preparations of crude extract are dialyzed in cellophane tubes, most of the active material remains in the dialysis sac, but enough active substance passes through the sac so that the dialysate prevents cleavage in Chaetopterus eggs. In general, therefore, we are led to conclude that the active principle of the starfish extract is a carbohydrate combined with protein. In view of the fact that the extracts are strongly metachromatic, the carbohydrate may well be an acid mucopolysaccharide of the heparin type.

K and Na content in dogfish leukocytes. H. G. HEMPLING.

Leukocytes of the smooth dogfish, *Mustelus canis,* were separated from the peripheral blood by mild centrifugation. The buffy layer was transferred to narrow tubes of 7 mm. diameter and 15 cm. length and mildly centrifuged again. The buffy coat was then re-suspended in either serum or elasmobranch Ringer's solution containing urea. The per cent distribution of cells obtained in this fashion were: small lymphocytes: 90.8; large lymphocytes: 3.7; thrombocytes: 2.8; eosinophils: 1.2; erythrocytes: 2.3. The cells were packed in the air turbine and, after obtaining wet weights, were cytolyzed in distilled water. Analyses were made on the supernatant, after removal of the residue, using a Beckman flame photometer. In 6 experiments, K values averaged 136.0 ± 13.8 meqs/kg. cell water. In 3 experiments, Na values averaged 88.7 ± 21.8 meqs/kg. cell water. Per cent water averaged 78.2 ± 1.0. Cells suspended in serum or in elasmobranch Ringer's containing urea, at 6° C. for seven hours showed no significant changes in total or differential count. K loss for this same period did not exceed 15% of the initial value. Cell water content remained constant. However, cells suspended in serum at 6° C. for two days showed a 92% decrease in total cell count.

The action of uranyl salts on the erythrocyte surface. JOSEPH F. HOFFMAN.

The effect of uranyl nitrate, acetate and chloride was studied on dogfish, tautog and human erythrocytes. The uranyl ion was found to be responsible since essentially the same result was obtained with each salt. Three types of phenomena were studied: (1) Clumping. Dilute suspensions of well-washed dogfish cells were clumped in concentrations of uranyl nitrate above 1×10^{-4} m/l.; the greater the concentration of uranium, the larger the average number of cells per clump. Clumping could be almost completely reversed by reducing the uranium concentration by dilution or by the addition of phosphate. Clumping occurred at lower concentrations of uranium in tautog and human than in dogfish. The rate of clumping could be measured by the densimeter technique used in permeability measurements. (2) Shape. In nucleated erythrocytes uranium induced changes in cell shape ranging from crinkling to crenation; with human cells, from cupping and crenation to leaf shapes. These changes occurred in, and were dependent upon, uranium concentrations which did not cause clumping. (3) Permeability. The rate of exit of urea from dogfish cells was found to be reversibly inhibited in proportion to the uranium concentration. The maximum inhibition was ca. 200% that of the control limited by clumping at any higher concentration. The entrance of urea into the cells (slower than exit under identical gradients) could be inhibited, but to a lesser degree. Control of the pH or the presence of plasma protein had no appreciable effect on the rate or the per cent inhibition. Uranium had no measurable effect on the rate of passage of water, thiourea, glycerol or ethylene glycol. Glycerol entrance could be slowed in human and tautog erythrocytes, but not ethylene glycol or water. Since these results can be interpreted as surface phenomena, uranium is pictured as combining (reversibly) with the plasma membrane, presumably the protein components.

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[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

On the reproductive rate of two algal species after treatment with growth-affecting substances. JOHN W. KING.

Two algal plants, *Chlamydomonas media* and *Sphaerella lacustris,* were grown for 18 days on a medium of Knop's solution in 2 per cent agar with a trace of growth-affecting substance. Before the plates were inoculated, the solution containing the algal cells was drawn into a Thoma mixing pipette and shaken for three minutes. This was done in order that approximately the same number of cells would be contained in equal volumes of algal suspension. One drop of suspension from the mixing pipette was then placed on the experimental and control plates. The diameter of the circles made by these drops was found to be 6 mm.

After eighteen days in diffused light, considerable increases in diameter size of these growth circles were observed. The increases were as follows:

	C. media	*S. lacustris*
Control	.1 mm.	.1 mm.
Indole acetic acid	.4 mm.	.4 mm.
Indole-3-n-butyric acid	.4 mm.	.3 mm.
Indole-3-n-propionic acid	.2 mm.	.2 mm.
Colchicine	.6 mm.	.5 mm.

Microscopic examination of the cultures showed that the increase in diameter of algal circle was due to an increase in the number of cells. Therefore it was concluded that the number of algal cells varies directly with the size of the growth circle. It was also observed that *C. media* grown on agar culture containing indole acetic acid or colchicine was much greener in color and more luxuriant in growth than was the control.

Efforts are now being made to determine the per cent of increase during a particular period by making cell counts and by the spectrophotometric method. In order to determine the incessancy of this change in reproductive rate, transfers from experimental media to a number of control media will be made.

The influence of pituitary posterior lobe preparations on the action of phenolases in vitro. ROBERT R. KOHN.

It has been shown by Frieden and Bozer, and others that there is an increase in melanin pigment in vertebrates when a posterior pituitary extract rich in intermedin or when ACTH with intermedin activity is administered. Experiments with various hog posterior lobe preparations and phenolases were carried out colorimetrically in an attempt to extend or negate Fostvedt's unconfirmed results on the direct action of posterior pituitary fractions on mealworm tyrosinase.

Aqueous and dilute acid extracts, and re-suspended acetone and ether precipitates of posterior lobe extracts had no significant effect on mushroom tyrosinase-tyrosine reactions. Extracts boiled at a high pH were usually strong inhibitors of the reaction. An effective preparation was made according to the method of Landgrebe and Waring for intermedin, in which the oxytocic and pressor activities were destroyed, the active principle adsorbed on charcoal and eluted with acetic acid. This preparation shortened the lag period and removed the inhibition of mushroom tyrosinase caused by glutathione. Inhibitions of 28% and 31% were completely overcome while uninhibited reactions were stimulated up to 15%. Natural copper inhibitors may have been present in the enzyme preparation. Adequate controls were maintained.

The active preparation overcame glutathione inhibition of a *Palaemonetes vulgaris* catecholase-catechol reaction, but did not remove the lag period. Inhibitions of 33% to 48% were 75% to 83% removed, and uninhibited reactions were stimulated up to 36%.

A catecholase extracted from *Loligo* skin was inhibited up to 43% by glutathione. Inhibitions were completely overcome by the active pituitary preparation but the lag period was not consistently shortened. Uninhibited reactions were inhibited by the same concentrations of active preparation which protected against glutathione.

Effect and significance of various steroids and of ACTH (corticotropin) on the cleavage pattern of Arbacia ova. VALY MENKIN.

Cortisone acetate (Merck) (2.5 mg. in 10 cc. of sea water) tends to suppress the incidence of normal cleavage in fertilized *Arbacia punctulata* ova. This is not referrable to the vehicle in which the steroid is suspended. The vehicle does not alter the cleavage pattern. Filtering the suspension of cortisone acetate yields a cortisone residue capable of suppressing the incidence of normal cleavage, whereas the filtrate, which is essentially the suspending vehicle, has also no such inhibitory effect. The sperm are likewise inactivated by cortisone acetate (2.5 mg. in 10 cc. of sea water).

Desoxycorticosterone acetate and the glucoside tend to have the same inhibitory effect on the cleavage of ova.

ACTH (corticotropin) (Armour and Organon products) directly affects the cleavage pattern of Arbacia ova. There is also a greater incidence of suppression. This indicates that this pituitary hormone can likewise have a direct toxic effect on the ova without the mediation of the adrenal cortex.

In contrast, a protein anabolic steroid, testosterone proprionate (Ciba), has no effect on the incidence of normal cleavage in Arbacia ova. Somatrofin (STH, a Horner product), a growth hormone from the anterior pituitary, has likewise no effect on the cleavage pattern.

The suppressing action of ACTH is not a protein effect and it is not due to any differential solubility of the hormone.

The specific results suggest that the suppression of cellular activity, as measured by cell division, of cortisone and ACTH throws light on the mechanism of suppression of these hormones in inflammation as reported in 1940 and 1942 by the writer.

Activation of eggs of Nereis limbata by a surface active extract of dead sperm. W. J. V. OSTERHOUT.

If air was passed through the suspension of live sperm in sea water at room temperature little or no foam was produced. But if this suspension was heated for ten minutes at 55° C., thus killing all the sperm, a highly surface active substance was released. The entire extract was converted into foam when air was passed through it at room temperature. When this foam was dissolved in sea water and suitably diluted (pH value unaltered from that of the sea water) it activated the eggs. In favorable cases practically all the eggs extruded full jelly in a few minutes. Substantial numbers of them extruded polar bodies and if the eggs were transferred soon enough to a larger quantity of sea water, segmentation to two- and four-cell stages was observed. Later some abnormal trochophores were found.

Each extract was examined and found to contain no live sperm. The control was made by treating the sea water without sperm in exactly the same way. This had no effect on the eggs. Care was taken to avoid rise in temperature sufficient to activate. Experiments were carried out at 21° to 24° C. within three hours after collecting.

Every precaution was taken to prevent contamination by live sperm. In no instance did the experiment show contamination. Only normal eggs were used.

The same results were obtained with the extract which had been freed from the dead sperm by centrifugation.

Activating substance passed through a dialyzer but the inside solution remained more effective.

As mentioned last October in this journal a surface active substance in the sperm may play a role in activation.

Reversible contraction of protoplasmic structures by changes in pH values. W. J. V. OSTERHOUT.

The chloroplasts of the fresh water plant *Nitella flexilis* J. Ag. shrink in striking fashion when cells are killed in 0.1 M HCl, but regain their original size if well washed in distilled water. The entire process is reversible and can be repeated back and forth many times on the same chloroplast. Certain polyelectrolytes act in this way, presumably because negative charges on the molecule, which repel each other and make the molecule larger, are neutralized by the addition of hydrogen ions.

Further studies on the growth of oysters under different ecological conditions in Rand's Harbor. MADELENE E. PIERCE AND JOANNE E. CURRIER.

Studies on the growth of oysters begun in the summer of 1950 were continued in the summers of 1951 and 1952, during a 16-week period from June 19–Oct. 12. The same stations, representing four diverse locations, were maintained.

Bottom salinities at all stations remain very uniform, 29–32 p.p.m. Surface salinities vary widely, 1–32 p.p.m., and are often very low. A precisely defined film of cold fresh water, 5–6 inches deep, overlies the warm saline water. This film is particularly marked in the widened upper ends of the arms although it may extend along the margins toward the outlet to Buzzards Bay. The water which forms the film comes not only from the brook flowing into each arm, but also from the seepage of cold springs (12.5° C.) along the shore. Since this film moves up and down with the tide, the oysters living in the intertidal zone are bathed in it for several hours.

Temperatures, except in the film, are uniform on any one day, but fluctuate with the season. The pH is uniform for the saline waters, 7.8–8.1, but drops to 6.5–6.9 in the film.

A comparison of the plankton with the stomach contents of oysters showed a reasonable correlation. At the three stations near fresh water inflow the dominating forms were *Melosira, Pleurosigma* and *Nitzschia*. Near the entrance to Buzzards Bay, the dominating forms were *Chaetoceros*, copepods and veligers.

Except for the station in the stream, all trays of oysters showed about equal total growth in 1951 (results for 1952 are not yet complete). There was a tendency for more rapid increases in length and width to occur in the early summer, while that of depth occurred in the later part. Increase in volume occurred, however, at a uniform rate throughout the season. These results are in contrast to those of 1950 in which two trays outstripped all others in total growth of all dimensions, and all trays showed a significant increase in volume during late summer and early fall.

Fibrous ultrastructure in the hypobranchial mucus of Busycon. R. R. RONKIN.[1]

Observation of the remarkable flow properties of mucus prompted this search for their structural basis at the submicroscopic level. Smears of mucus from the hypobranchial gland of *Busycon canaliculatum* (L.), a prosobranch mollusk, were prepared on collodion films, dried in air, shadowed with chromium and photographed with the electron microscope at 7720 ×. Ropy strands pulled out of masses of fresh mucus were treated in the same manner. Dimensions in microns (means and their standard errors) are given for the characteristic structures observed.

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[1] Aided by the Research Committee of the Faculty of the University of Delaware and by a contract between the Office of Naval Research, Department of the Navy, and the University of Delaware (160-015).

Smears contained large elliptical bodies (length, 1.06 ± .070, $n = 5$; width, .28 ± .025, $n = 6$), some with fine transverse striae (spacing, .022 ± .0015, $n = 13$); an abundance of irregular, somewhat spherical bodies (diameter, .074 ± .0021, $n = 50$) with irregular surface sculpturing, and masses of oriented fibers of indeterminate length (diameter, .028 ± .0026, $n = 7$). Preparations of **strands** contained irregular bodies like those just described and broad, clearly defined ribbons of indeterminate length (diameter, 1.40 ± .27, $n = 6$). Margins of ribbons bore double edges, appearing as two opaque lines .037 ± .0038 ($n = 10$) apart, separated by a clear space. Some ribbons appeared to contain longitudinally oriented fibers of about the same diameter (.029 ± .0025, $n = 10$) as those found in the smears. The fibrous ultrastructure in both types of preparations may be significant in the interpretation of the mechanical properties of mucus and the birefringence of fresh mucus strands. No explanation is offered for the other structures observed.

Notes on the circulation in the yolk sac of the toad fish, Opsanus tau.[1] PAUL G. ROOFE.

The blood supply to the yolk sac is through the vitelline artery which is located in a more or less sagittal position posteriorly and ends frequently in bilateral branches which form a loop at the base of the yolk sac. The anterior convergence of this loop is the beginning of the vitelline vein which runs dorsally in an anterior sagittal position, ending in the sinus venosus. Between these two large vitelline vessels, numerous connecting links occur; some are true arteriovenous anastomoses; others are arterioles ending in capillary loops which converge to form venules which in turn enter the vitelline vein. The arterial twigs end in truncated cones as does the vitelline artery. The flow of blood is non-laminar, that is, it is turbulent. This turbulence has the appearance of a true "sludge," using Knisely's terminology. The basic units are two or three agglutinated red cells. The embryos are thrown into violent "spasms" of *S*-shape movements due to the accumulation of metabolic products in the internal environment. After each of such episodes, the turbulence of the blood is increased. Periods of inactivity allow the turbulence to clear up. The reason for the agglutination and its partial disappearance is unknown.

Responses of the distal retinal pigment of Crago septemspinosus to changes in illumination. MURIEL I. SANDEEN, MILTON FINGERMAN AND FRANK A. BROWN, JR.

The distal retinal pigment of the sand shrimp, *Crago septemspinosus,* shows active movements in response to changes in illumination. The normal time for maximal dark adaptation is about 90 minutes upon transfer from light to darkness and the time for complete light adaptation when animals are transferred from darkness to 250 ft. c. is about 30 minutes. The pigment also exhibits a smoothly graded relationship between the degree of its distal migration within the pigment cells and the logarithm of illumination over the range from about 0.00002 ft. c. to about 10 ft. c. Above 10 ft. c. no further light-adaptational response of the pigment is evident. There is also a distal pigment response to the shade of background when incident illumination is constant. The pigment is in a somewhat more dark-adapted position when the animal is upon a black background than when upon a white one. The response to background is not an albedo one, but is entirely explicable in terms of the influence of the background in determining the amount of light reaching the great majority of the ommatidia of the eye. Dark-adapted *Crago* respond to a one-minute, 250 ft. c. flash of light by an extensive but transitory light-adaptational response of the distal pigment, which reaches the fully light-adapted state in about 30 minutes, remains there for about another 30 minutes, and reaches the maximally dark-adapted state again only after four to five hours. This prolonged response to a brief light stimulus is interpreted as evidence for the abrupt discharge of a large amount of light-adapting hormone into the blood upon dark-to-light change.

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[1] This work was aided by a grant from the Office of Naval Research, N6 ori T. O. 1.

The prevention of the iodoacetate effect on the sodium and potassium content of Ulva lactuca by photosynthesis. George T. Scott, Hugh R. Hayward and William D. Andrus.[1]

The influence of the glycolytic inhibitor, monoiodoacetic acid, on the sodium and potassium balance in living cells has been investigated by Wildbrandt (1940) on erythrocytes, Dean (1940) on muscle, Dixon (1949) on brain cortex and Scott (1951) on baker's yeast. An interpretation of the action of this agent can be based on its inhibitory effect on carbohydrate metabolism which is associated with ion transport.

The influence of monoiodoacetate on *Ulva lactuca* follows essentially a similar pattern resulting in a loss of K^+ and a gain of Na^+ under conditions of darkness. In the presence of sodium iodoacetate (0.001 M) for 24 hours the potassium content falls from 32 to 6 milleq./100 g. cell water, or to 20% of that of the controls. The Na^+ content increases from 11 to 30 milleq./100 g. cell water, which represents a 100% increase in "internal" Na^+. Time-course curves indicate a more rapid rate of ion transfer in material which had been kept in the dark for 24 hours prior to the experiment.

When the alga is maintained in the light during the course of the experiment, no loss of K^+ or gain of Na^+ compared to the controls occurs over a 24-hour period. Photosynthesis prevents the iodoacetate effect on these electrolytes.

A probable explanation of this observation is that the energy for ion transport and for the maintenance of normal Na^+ and K^+ balance is derived from the utilization of phosphoglyceric acid. Iodoacetate prevents the formation of this intermediate by its inhibition of phosphoglyceraldehyde dehydrogenase.

Under conditions of illumination photosynthesis provides the cell with phosphoglyceric acid which, according to Gaffron (1951), is the important intermediate in carbon dioxide reduction; hence, even in the presence of the inhibitor this material is available to cellular metabolism.

The rate and extent of exchange of K^{42} with cellular K^{39} in Ulva lactuca. George T. Scott, Hugh R. Hayward and William D. Andrus.[1]

Experimental evidence obtained principally on muscle and erythrocytes has shown that under physiological conditions a steady-state prevails in which the uptake and loss of K^+ are equal. By means of K^{42} it is possible to obtain information on this "equilibrium" state. The green alga, *Ulva lactuca,* offers favorable material for an extension of such investigations, since it consists of membranous fronds two cell layers in thickness, hence permitting a precise measurement of ion exchange between the cells and the surrounding sea water medium.

Small uniform discs of a frond were placed in sea water containing a tracer amount of K^{42} and maintained at three different temperatures in the light and in the dark. At various time intervals samples were removed, rinsed briefly in isotonic sucrose, weighed and counted for radioactivity. The material was then analyzed chemically for K^+ content by flame spectrophotometry so that specific activities could be calculated. The specific activity of the sea water was also determined.

These data indicate that the specific activity of the algae, $\frac{[K^{42}\text{ inside}]}{[K^{39}\text{ inside}]}$, reaches equality with the specific activity of the sea water medium, $\frac{[K^{42}\text{ outside}]}{[K^{39}\text{ outside}]}$; hence all the K^+ in the cell is exchangeable.

In the light at 10° C. 90% of the K^+ is exchanged in 4.4 hours; at 20° C., in 2.8 hours; and at 30° C., in 0.6 hour. Under conditions of darkness the rate of exchange is significantly less than in the light. Semi-log plots of the time course curves indicate essentially an exponential uptake of K^{42}.

The technique of photomicroscopy with the Polaroid Land camera. D. R. Shanklin.

When a concomitant record of the progress of an experiment is desired, conventional photomicroscopy being inadequate, the Polaroid Land camera is adaptable. The microscope used was an AO MH35 with oculars of various designs and manufacture giving 22.5 to 1405 diameters. A B&L student microscope was satisfactory, as was half of a binocular dissecting microscope. The technique follows: (1) the microscope is focused on the desired field, either vertically or horizontally; (2) condenser and diaphragm are set to desired light effect; (3) the light source is masked so that the only direct rays to reach the face of the camera do so through the microscope; (4) the camera is set on *bulb* exposure; (5) bellows are fully extended to the 3½ focus notch; (6) camera is adjusted so that the microscope centers a pencil of light through the diaphragm of the camera; (7) the pencil is focused by moving the camera to its smallest point on the surface of the shutter; (8) the smallest opening that will admit all of this pencil is set, usually No. 7 or 8, thus assuring sufficient light-tightness; (9) shutter lever is depressed for desired time; (10) activate prints for 60 seconds by standard Polaroid technique and observe results. Some times and situations that gave satisfactory results follow: 45 ×, 3 sec.; 97.0 ×, oil immersion, 15 sec.; 430 ×, dark field, 90 sec.; 1405 ×, wide field, 60 sec. Stronger light sources that gave readings on the light meter gave satisfactory results at the indicated *snapshot* shutter speeds. If one is willing to sacrifice one roll in adapting to his own microscopic equipment, he may find, as this worker has found, a valuable tool whose expense is palpable at reasonable application.

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[1] Aided by a contract between the Atomic Energy Commission and Oberlin College.

The effect of centrifugation and low temperature on development of Fundulus heteroclitus.[1] J. R. Shaver and Susumu Ito.

Previous studies have shown that centrifugation of eggs of *Fundulus heteroclitus* for 30 minutes at 300 g during the first 30 minutes after insemination produces a spectrum of abnormalities the most striking of which is duplication of parts or of the whole embryonic axis. The latter effect occurs in a small number of cases (ca. 1.0%) and it was thought that a combination of centrifugation and low temperature might increase the incidence of duplication, since the latter agent has been reported to produce this result in Fundulus. Eggs were centrifuged at room temperature over 0.83 *M* sucrose at 300 g for 30 minutes, beginning 10–22 minutes after insemination, placed immediately in sea-water at 4–6° C., and maintained at this temperature for 20–24 hours. Batches of eggs were concomitantly placed at room temperature, centrifuged and placed at room temperature, or subjected to the low temperature treatment. Mortality of embryos after the double treatment was 20% higher than that due to either agent alone, and the numbers of normal embryos were correspondingly lower. Numbers of abnormal embryos were not significantly different in the three experimentally treated lots. No duplications were obtained after exposure of eggs to low temperature alone. Twinning of parts or of the whole axis after centrifugation, or after centrifugation plus low temperature treatment, varied in different experiments, but the total percentages of these effects after the two types of treatment were not significantly different, and were of the same order as had been obtained by centrifugation alone (1.0–2.0%). A preliminary study of abnormal and duplicated embryos in section indicates deficiencies in embryonic organization and differentiation similar to those produced in amphibian embryos by centrifugation. The results of these studies are interpreted as indicative of an early formation of an organization center in the Fundulus egg comparable to that in amphibian eggs, the experimental alteration of which may result in some cases in splitting and axial duplication.

Survival of Spisula (Mactra) ova. Victor Schechter.

Investigation of survival of unfertilized egg cells of the clam, Spisula, was made during the summer of 1952, in sea water of 23 to 24° C. In contrast to the results of experiments previously performed at temperatures of as much as seven degrees lower, it was generally found that cytoplasmic deterioration preceded germinal vesicle breakdown. Vitamin K (2-methyl-1,4-naphthoquinone), a coagulation factor, reduced survival time, even in concentrations as low as ⅕₀ milligram per 100 cc. of sea water. It was not found possible to stay this effect with either heparin or hirudin. Hydroquinone, in comparable concentrations, also shortened the life span, but the response of the egg differed in certain respects from that to vitamin K. Urea, known as a protein-lysin in higher concentrations, had a slightly beneficial effect in one per cent solution. However, in combination with vitamin K, it increased the destructive effect of the latter. Dilution of the sea water medium produced various effects at different concentrations. The eggs swelled, demonstrating an intake of water. The egg membrane showed a capacity for stretching from approximately 7500 to more than 10,000 square microns before rupture occurred. In fresh water or in 10 per cent sea water bursting resulted in a few minutes; 40 per cent sea water caused rupture of the egg cortex and some loss of cytoplasm, followed by healing and fusion together of cells which happened to be in contact. At about 50 per cent concentration survival time was greater than in undiluted sea water, with an optimal point at approximately 70 per cent.

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[1] Aided by Summer Research Grant, University of Missouri.

Effect of pH on "resting" Tetrahymena. JOHN V. SLATER.

Using a veronal-acetate buffer system it was found that "resting" cells of *Tetrahymena geleii* E. (approx. 2-4000/ml.) were able to tolerate a pH range of 6.4–9.9 for a period of 24 hours. When acid buffers were used (pH 3.7–5.8), the ciliates were killed but not cytolyzed. Values higher than 9.9 dissolved the cells. Stationary phase animals placed in buffers of pH 9.5 and higher (to 10.9) adjusted the pH downward. Animals killed in the acid range did not change the hydrogen ion concentration. Homogenates of stationary phase cells, of about the same number of animals as used in the tests, had no effect on buffers of high pH. Very heavy suspensions, however, when homogenized did lower the pH of buffers set at 9.6. The internal pH of this ciliate, as determined with homogenates, is 6.68. It is believed that living stationary phase cells, in the absence of the full nutrient spectrum, do appreciable work within a short period of time in adjusting the pH of their external environment.

Neural tube extirpation in Fundulus heteroclitus and resultant neural arch defects. RAY L. WATTERSON.

The role of the neural tube in neural arch formation is undergoing investigation in amphibians (Holtzer) and birds (Fowler and Watterson) by extirpation and transplantation experiments; this study extends the investigation to fish.

Attempts were made to extirpate a length of neural tube as cleanly as possible at Oppenheimer's stage 18 (4–14 somites); embryos were sacrificed after neural arch development was evident at unoperated levels. Operated specimens lacked spinal cord for a distance of 18–297 micra just posterior to the brain. In controls neural arches occur every 63–72 micra so longest extirpations covered a distance of at least four pairs of neural arches.

Results: (1) Typically no cartilage cells can be found lateral or dorsal to notochord at level of extirpation if nervous tissue is entirely lacking; (2) simultaneously with appearance of spinal cord anterior and posterior to level of extirpation cartilaginous neural arches appear; (3) if a small stretch of nervous tissue is present anywhere within level of extirpation, cartilage cells are usually associated with it; (4) badly disorganized nervous tissue can have relatively normal neural arches associated with it; (5) neural arches can in some instances be complete in dorso-ventral extent even though the dorso-ventral extent of the spinal cord is reduced appreciably; thus all dorsal skeletogenous cells which arise do not have to be in direct contact with nervous tissue.

Results are compatible with interpretation that the neural tube induces the somites to provide cartilage cells for neural arch formation and that the sclerotome does not arise by self-differentiation of the somite.

Occasional small clusters of cartilage cells lie dorso-lateral to the notochord in position of bases of neural arches in absence of spinal cord; these are usually closely related to adjacent haemal arches and presumably arise from them. Other clusters lie close to dorsal pigment cells.

The influence of light intensity and temperature on chromatophores of Palaemonetes vulgaris. H. MARGUERITE WEBB, FRANK A. BROWN, JR. AND ROBERT C. GRAVES.

The ability of *Palaemonetes* to respond to a white to black background change at light intensities ranging from 3800 to 0.00031 ft. c. was investigated by observing the response after one hour. The maximum dispersion of the red pigment was obtained at 0.1 ft. c. with decreasing dispersion up to 3800 ft. c. at which intensity, however, the pigment was still somewhat dispersed. Between the light intensities of 0.1 and 0.00031 ft. c. there was an abrupt reduction in amount of dispersion until at 0.00031 ft. c. the red pigment was as concentrated as initially on the white background. The degree of dispersion of the white pigment on both the black and white backgrounds varied directly with the logarithm of the illumination from 0.1 to 3800 ft. c. Below 0.1 ft. c. the white pigment was maximally concentrated.

The chromatophore responses at a series of temperatures from 5° to 35° C. were also studied. The animals were permitted to adapt to the background for one hour and then to adjust to the temperatures for one hour. On a black background the degree of dispersion of the red pigment was greatest at 20° C. and became less as the temperature decreased to 5° C. and increased to 35° C. On a white background the pigment was maximally concentrated at 20° C and 25° C. and tended to become more dispersed as the temperature decreased to 10° C. and increased to 35° C. At 5° C. the red pigment of the animals on the white background was found to be more concentrated than at 10° C. The white pigment behaved randomly with respect to temperature, with the pigment partially dispersed at all temperatures irrespective of background.

Environmental factors concerned with the lethality of x-rays in Paramecium. RALPH WICHTERMAN AND FRANK H. J. FIGGE.[1]

Specimens of *Paramecium caudatum*, cultivated on the bacterium *Aerobacter aerogenes*, were irradiated with two cross-fired x-ray tubes (180 kv. pk., 25 ma, no filter, intensity 7420 roentgens per minute). Temperature was determined during irradiation by means of a thermojunction placed in one of the control irradiation chambers. X-ray survival curves for microorganisms as reported in the literature vary, apparently depending upon the conditions employed for irradiation. We find, for instance, that under certain conditions the LD 50 may vary from 75 kiloroentgen to 350. The present work attempts to analyze the cause of variability and establish a standard, repeatable method. The ratio of the number of paramecia to volume of culture fluid is not nearly as important as the ratio of volume of air to the volume of culture fluid in plastic irradiation chambers of various sizes and shapes. Lethality of x-rays in Paramecium is related not only to the gradual accumulation of a toxic concentration of hydrogen peroxide and other similar compounds in the irradiated medium—a fact established long ago by many investigators using a variety of microorganisms—but our evidence indicates that it is also greatly influenced by a toxic substance coming from the air-space of the irradiation chamber. The rate of diffusion into the culture fluid depends on the surface area of the medium and its depth and volume. Attempts are being made to identify the toxic principle which may be derived from the oxygen in the air of the chamber, ozone or some other gaseous substance diffusing into the aqueous medium.

To eliminate factors which cause variation in survival curves, a 2-cc. nylon hypodermic syringe was used as an irradiation chamber. This device absorbs very little irradiation, eliminates air from the irradiation chamber and permits the introduction of various substances to be tested during irradiation. Accurate sampling of specimens after intervals of irradiation without changing the depth of the medium is also a desirable feature. It is thus ideal for the study of lethality of x-rays in Paramecium and may prove useful for similar studies with other microorganisms.

Effect of respiratory inhibitors on melanophoral activity of frog skin in vitro. PAUL A. WRIGHT.

In attempting to discover something of the nature of energy sources for melanophoral activity, a study was made first of oxygen consumption in excised pieces of frog skin (*Rana pipiens*) as detected in Warburg respirometers. In four tests, squares of blanched skin in Ringer's solution consumed 21.3 mm.^{3} oxygen per cm.^{2} per hour at 25° C., and no change in rate was observed when intermedin was added, even though darkening occurred as usual. In four subsequent tests, darkened skin in dilute pituitary-Ringer solution consumed oxygen at the same rate, which was again unchanged when blanched by addition of a concentrated adrenalin solution.

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[1] This work was supported by the American Cancer Society (Maryland Div.), Comm. on Research, Temple Univ., and O.N.R., Dept. of the Navy.

Using the photoelectric method for detection of color change in excised skin, a series of respiratory inhibitors was tested for possible activity in inhibiting either darkening or blanching. KCN (10^{-3} to 10^{-5} M), NaIAc (10^{-2} to 10^{-4} M), Na_3N (10^{-2} to 10^{-4} M), urethane (10^{-1} to 10^{-3} M), or NaF (10^{-3} to 10^{-5} M), combined with 0.05 frog pituitary in 40 ml. Ringer's, had no effect on the darkening curves, nor was subsequent blanching in Ringer's solution delayed. Skins darkened by intermedin and then placed in the same concentrations of inhibitors blanched at the same rate as controls placed in Ringer's solution alone. Assuming that the inhibitors penetrated adequately, it appears that enzymatic energy-releasing reactions, which in other situations are inhibited by these chemicals, are not involved in melanophoral activity. These results bear a special significance in view of complete inhibition by all of these substances in appropriate concentration of ovulation *in vitro* in the frog recently reported by Rondell. Although the processes of ovulation and color change may not be considered wholly comparable, they are both pituitary-induced reactions, and the opposing results have been obtained under practically identical experimental conditions *in vitro*.

Effect of crustacean eyestalk hormone on melanophore activity in excised frog skin.

PAUL A. WRIGHT AND ROBERT R. KOHN.

It has been reported by Abramowitz, Perkins and others that extracts of the eyestalks of several crustaceans are effective in darkening the skin of hypophysectomized frogs, and on the basis of these results and reciprocal data showing the appropriate response in Crustacea upon injection of vertebrate pituitary extract, an hypothesis has been proposed that the crustacean chromatophore hormone and vertebrate intermedin are structurally very similar if not, indeed, identical. Our experiments with excised frog skin (*Rana pipiens* and *R. clamitans*), using the photoelectric method for detection of color change, have led us to a wholly different conclusion. Of 46 tests in which extracts of eyestalks from *Callinectes, Palaemonetes* and *Uca* in dosages from the equivalent of 1 to 40 stalks in 40 ml. Ringer were used, one (10 *Palaemonetes* eyestalks) showed a response comparable to that produced by 0.05 frog pituitary in the same volume of fluid. This experiment could not be repeated, and the positive result may likely have been due to contamination of glassware with frog pituitary. Five other experiments using 6 and 10 *Uca,* 10 and 20 *Palaemonetes,* and two *Callinectes* eyestalks gave responses equivalent only to that produced by 0.002 frog pituitary in 40 ml. Ringer, and therefore can hardly be called conclusively positive. All other extracts elicited no darkening of frog skin whatsoever. Triturated eyestalks were extracted in Ringer's solution or in 0.7% NaCl, with and without boiling, and their activity for crustacean melanophores was ascertained by the fact that of several tested on eyestalk-less *Palaemonetes,* all gave positive results. Our data leave the impression, then, that the eyestalk hormone and intermedin are physiologically quite dissimilar, and raise the question whether positive results in hypophysectomized animals may not have been brought about through some other pathway than direct action of eyestalk hormone on amphibian melanophores.

Effect of colchicine on melanophoral activity in excised frog skin (Rana pipiens).

PAUL A. WRIGHT AND JAMES SABAL.

The fact that colchicine arrests chromosomes in metaphase and also potentiates the effects of several classes of hormones, has prompted the investigation of its effect on melanophoral activity because this process involves, like chromosomes in mitosis, movement of a kind of cellular particulate, *i.e.*, melanin granules, and is under hormonal control by way of a secretion from the intermediate lobe of the pituitary gland. Exposure of excised blanched frog skin to 10^{-3} or 10^{-4} colchicine in Ringer's solution does not darken the skin nor affect in any way its ability to darken when immersed in intermedin solution, as determined by readings taken in a photoelectric colorimeter. However, subsequent blanching, which would normally follow upon transferring skin from intermedin solution to Ringer's solution, is almost totally inhibited, and melanophoral pigment remains permanently dispersed. Darkening occurs normally when skin is exposed to pituitary solution to which 10^{-3} or 10^{-4} colchicine has been added, but, again, blanching does not follow in Ringer's solution. If pituitary and colchicine are combined one hour or more in advance of immersing the skin, partial (50–95%) inhibition of darkening may be observed and the melanophores are then refractory to intermedin alone.

Temporary blanching of a skin which has been darkened by a pituitary-colchicine mixture may be induced by adrenalin solutions, but melanophoral pigment disperses again after the adrenalin has washed out of the skin.

Colchicine is ineffective in preventing the blanching of a skin if it is applied after the skin has been darkened by intermedin alone. It appears, then, that colchicine enters into some sort of chemical combination with intermedin which prevents normal blanching of the skin. If colchicine and intermedin are allowed to react over a period of an hour or more, a compound may be produced which will partially inhibit darkening of the skin as well.

The bearing of new results in protein structure on cytological interpretations.[1] DOROTHY WRINCH.

Recent results suggest that the ribonuclease structure and the horse myoglobin and hemoglobin structures are made up of the same type of relatively small globulite molecules (20–25 Å across), in various numbers, various situated and various substituted (Wrinch, 1952). Following the general belief that globular proteins have a common type of structure, the cytoplasmic proteins, also, would be expected to comprise this same kind of molecule. In this event, it would be necessary to revise certain cytological interpretations. There are, in cytoplasm, systems of interlinked protein structures and other molecules which undoubtedly form continuous networks through various parts of or even through the whole of the cell. At present the protein fibrils therein are pictured as bundles of polypeptide chains, the protein in plasma membranes as such chains set in sheet-like arrangements. With the new type of structure for the proteins, *flexible three-dimensional networks* of proteins may be pictured (Wrinch, 1940). An immense variety of networks of globulite molecules, with various connexities and various water contents, can be worked out, capable of filling sphere-like or sheet-like or rod-like spaces. The difference between the two interpretations lies at the Å and $\mu\mu$ levels, the levels at which solutions of structural problems of cytology, such as the nature of cell permeability, have to be sought. The nature of the difference is best understood when we contrast a parallel array of polypeptide chains of atoms as the explanation of protein fibrils on the earlier view with the newer picture of such fibrils as chain-like arrays of essentially globulite protein molecules.

Recent results regarding the structures of the globular proteins.[1] DOROTHY WRINCH.

The new phase of the work on protein structure in the light of crystal data is based on x-ray studies of horse hemoglobin (Perutz, 1949) and myoglobin (Kendrew, 1950) and ribonuclease (Carlisle and Scouloudi, 1951). It consists in a comparative study of the intensity data and of the vector maps, maps concerned not with atomic positions but with the distribution of vectors between pairs of atoms in the crystal. Using results obtained in studies of the languages of intensity space (Wrinch, 1946) and vector space (Wrinch, 1939) we now have an apparently reasonable preliminary interpretation which provides definite indications regarding the type of molecules in the three structures. In each case the molecules are (1) cage-like in form, (2) globulite in shape and (3) relatively small in size, say 20–25 Å across, the skeletons of the molecules, presumably polymers of interlocked NCC backbones, being say 12–15 Å in diameter. From these dimensions, it follows that each structure contains a different number of molecules, perhaps 12 for the hemoglobin and three for the myoglobin and two for the ribonuclease structure. If these results are a guide to globular proteins in general, we would expect larger proteins to have more molecules and smaller proteins fewer molecules in their structures. In this event, sets of functionally related proteins in different species with the same MW may each be examples of some particular network, for example the hemoglobins of a 12-molecule network, and the myoglobins of a three-molecule, and possibly related, network. In any case, the variation in networks suggests a new aspect of the immense variety of the biologically active globular proteins.

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[1] This work is supported by the Office of Naval Research under a contract with Smith College.

REPORTS ON LALOR FELLOWSHIP RESEARCH

Nucleoprotein complexes of sperm nuclei. JAY BARTON II.

Further study of the nucleic acid fraction of sperm nuclei resistant to removal by DNase has led to the following observations: (1) The fraction may be removed and recovered as a nucleoprotein complex by extraction of the DNase-digested nuclei with 2 *M* NaCl. (2) After removal of basic proteins by 0.2 N HCl, the resistant DNA can be easily removed by DNase.

Similar fractionation of the nucleus can be made by 2 *M* NaCl. Part of the nucleic acid of the sperm nuclei can be readily removed by cold NaCl. The remainder is removed only with difficulty if at all. The amounts of the removable fractions are as follows: Asterias, 72%; Mytilus, 73%; Mactra, 35%. These figures may be compared to the DNase-removable fractions: 85%, 84%, and 40%, respectively.

The DNA remaining after extraction with NaCl is readily removed by DNase. It is also removed by phosphate buffer alone. The phosphate extract is not a true solution but appears to be composed of fairly large particles dissociated from the remainder of the nucleus. The extract resists sedimentation at 19,000 RFC, but is brought down by 105,000 RFC. The DNA of the phosphate extract exists in a nucleoprotein complex very similar to that of the DNase-resistant complex in composition, though not in solubility.

These experiments distinguish between two of the nucleoprotein complexes of the nucleus: the first, widely studied, readily available complex of DNA and basic protein; the second, a physically separate fraction of DNA-protein characterized by its resistance to dissociation in strong salt solutions. The second fraction is not intrinsically resistant to DNase. Removal of the basic protein allows the enzyme to act. The resistance to removal by DNase can be interpreted as due to the spatial orientation of the DNA within the nucleus or to the unique mode of combination with proteins of the nucleus.

Studies on the distribution, potassium activation and mechanism of pyruvic phosphoferase. P. D. BOYER.

Pyruvic phosphoferase (which catalyzes the transfer of phosphate from 2-phosphopyruvate to ADP) from rabbit and rat muscle requires K^+ for activity. In the present studies, activation and distribution of this enzyme were measured by colorimetric determination of pyruvate appearance in a mixture containing enzyme, phosphopyruvate, ADP, Mg^{++}, K^+, purified yeast hexokinase and glucose, together with control measurements lacking ATP or K^+ or with Na^+ added in place of K^+. Pyruvic phosphoferase was found to be present and to show K^+ activation in all tissues examined including the following: muscle tissues from the marine forms skate, torpedo, Fundulus, Limulus, Pecten, Thyone and Phascolosoma; the fresh water forms Anodonta and Unio; rabbit heart, liver, kidney, uterus and brain; skate brain; and whole Tetrahymena. The enzyme from Anodonta and Unio showed considerably lower requirement for K^+ than the enzyme from marine forms or the rabbit. Kinetic studies showed that the inhibition of the enzyme activity by Na^+ and Li^+ did not conform to a simple competitive inhibition.

Measurement of exchange reactions between phosphopyruvate and pyruvate-2-C^{14} with a purified rabbit muscle preparation showed incorporation of radioactivity into the phosphopyruvate in the presence of enzyme, K^+, Mg^{++}, phosphopyruvate, pyruvate-2-C^{14} and ATP. The rate of reaction at equilibrium was only approximately $\frac{1}{4000}$ of the initial rate of phosphate transfer to ADP. The phosphorylation of pyruvate by ATP is likely the limiting step in the incorporation of pyruvate into muscle glycogen. Exchange was dependent upon the presence of ATP, thus ruling out the possibility that the reaction proceeds by formation of an enzyme-phosphate intermediate which subsequently reacts with ADP. The velocity of phosphate transfer to ADP is not decreased by ATP, which suggests that the combination of the enzyme with ATP is much weaker or independent of the combination with ADP.

Studies on the nonsaponifiable fraction of lipid material from marine animals. II. LEON S. CIERESZKO.

Cholesterol was isolated from the nonsaponifiable matter of the sand dollar, *Echinarachnius parma.* It was found that only traces of glyceryl ethers were present. Both these findings fit in with those of previous workers on the few other echinoids studied. The echinoids differ from the asteroids in that they contain cholesterol, while the latter possess stellasterols and the glyceryl ether, batyl alcohol, as major constituents of the nonsaponifiable matter.

Batyl alcohol was found to be absent from the skin fat of the common starfish, *Asterias forbesi.* The nonsaponifiable fraction of the skin fat contained about 10% of a substance which gave stable adducts with urea. This substance is presumably aliphatic.

The sterol of the colonial tunicate *Amaroucium constellatum* yields an acetate melting at 120–121°, and appears to be different from cholesterol. Cholesterol has been isolated from *Styela plicata,* the only tunicate previously examined for sterols. *Molgula manhattensis* also appears to contain a sterol not identical with cholesterol.

The oxygen consumption of squid nerve. C. M. CONNELLY.

A continuous flow respirometer utilizing the oxygen cathode has been employed to measure the oxygen consumption of stellar nerves of *Loligo pealii,* at rest and during activity, at 16° C. Giant axons of 400–450 μ diameter, stripped of accompanying small fibers of the stellar nerve, consume at rest 68 mm.^{3} of oxygen/gm.hr. (range 47–86, 8 nerves) (grams, wet weight). Whole stellar nerves, including giant and small fibers, have a resting consumption of 74 mm.^{3}/gm.hr. (range 62–95, 5 nerves).

Eight giant axons stimulated 50 or 100/sec. for 20 to 30 minutes showed during the first period of activity measurable increases to new levels of oxygen consumption, followed after activity by recovery to the previous resting level. Average increases were: for stripped giant axons conduction 50/sec., 3.7 mm.^{3}/gm.hr. (two experiments); conducting 100/sec., 4.2 mm.^{3}/gm.hr. (three experiments); for giant axons selectively stimulated in whole stellar nerve, the increases were 60% greater (three experiments). These axons stimulated for a second period or other axons stimulated for the first time showed much smaller or no measurable increase; in other nerves recoveries following increases were absent or incomplete.

Increases in the oxygen consumption associated with low-frequency activity in the small fibers of the stellar nerve are reproducible in the same nerve and are maximal in the range 29 to 43 mm.^{3}/gm.hr. (three nerves) at stimulation frequencies of 2 to 6/sec.

The increase in oxygen consumption per gram of nerve per impulse is thus many times (probably more than 50 ×) greater in the small fibers than in the giant axons. Studies of fiber size are being made to permit a quantitative test of the hypothesis that increase in oxygen consumption per gram per impulse is proportional to the surface area of the active fibers.

Comparative studies on ectodermal scleroproteins. MARCEL FLORKIN.

Electron microscopical studies pursued in collaboration with Dr. Ch. Grégoire have shown that the protein matrix of mother-of-pearl in molluscan shells is made of lace-like protein leaflets 2–4 mμ thick in gastropods and lamellibranchs and 4–12 mμ thick in the cephalopod *Nautilus.* Amino acid determinations, made in collaboration with Dr. G. Duchateau and Mr. H. Sarlet have shown that the protein concerned differs from the sclerotin of arthropod exoskeletons by a higher content of alanine and glycine and a smaller content of glutamic acid, histidine, isoleucine, lysine, proline, threonine, tyrosine and valine. On the other hand, while the water-soluble protein component of the lobster exoskeleton, arthropodin, shows the same composition as the scleroprotein of the same structure, sclerotin, the water-soluble component of nacre protein matrix shows a composition definitely different from that of the scleroprotein component of the same structure. These facts call for a comparative study of scleroproteins of ectodermal origin, in different animal groups. A number of materials collected in Woods Hole have proven suitable for such studies. Among them are the cuticles of *Mactra solidissima Busycon canaliculatum, Phascolosoma gouldii* and *Limulus,* and also the protein components of the tubes of *Diopatra cuprea* and *Chaetopterus variopedatus*. Separation experiments have been performed on these materials and sufficient amounts have been collected for parallel electron microscopical and chemical studies.

Some aspects of blood coagulation in certain decapod crustacea. PAUL J. FREEMAN.

An attempt was made in this laboratory to choose from among representatives of the decapod crustacea varying type blood-clotting systems and determine whether the noted differences between them were qualitative or quantitative in nature. Also, comparisons were made between crustacean clotting and that of the better known vertebrate system. The methods used were as follows: 1. Factors known to be essential to vertebrate clotting were introduced separately into the non- or weakly clotting crustacean clotting system in an attempt to induce firm plasma clotting. A variation of this method was to use vertebrate blood with an essential clotting factor removed which was then added to crustacean blood. 2. Dilution of a firm clotting crustacean blood, *e.g.*, *Callinectes sapidus*, with saline solution to a point of no clotting. This was then used as a control to compare dilutions made with non-clotting systems to determine whether clotting would occur at greater dilutions or not. 3. Use of anti-coagulants known to be specific in preventing essential vertebrate clotting factors from acting were tried on crustacean blood. 4. Comparative hematocrit determinations. 5. Observation of morphological changes in coagulocytes as clotting progressed.

The animals investigated may be divided into two major groups on the basis of their clotting reaction. Group I. No plasma clotting. This includes *Libinia*, *Carcinedes* and *Cancer*. Varying degrees of coagulocyte action were observed. Group II. Plasma clotting. This includes *Callinectes*, *Ovalipes*, *Homarus* and *Uca*. Coagulocyte action takes place first, followed by plasma coagulation.

The group I forms reveal no incipient plasma clotting factors such as coagulation enzymes or proteins. Hence, the differences in clotting reactions between the members of groups I and II appear to be qualitative in nature. *Callinectes* and *Ovalipes* reveal all the characteristics of vertebrate clotting. Sodium hydrosulphide prevents any coagulocyte action from occurring in the blood of members of both groups as revealed by absence of morphological cell changes and plasma clotting. This points to specialized blood cells as being the key factor in initiating all types of coagulation in decapod crustacea.

Effect of temperature on the birefringence of the mitotic spindle. SHINYA INOUÉ.

The birefringence of the metaphase spindle of the oöcyte of *Chaetopterus pergamentaceus* can be abolished when the temperature is either lowered below 4–5° C. or raised above 30–35° C. Upon returning the preparation to intermediate temperatures, a smaller spindle reappears, almost immediately, at the position where the original spindle had disappeared. At 25° C., the new spindle regains the full size in approximately 15 minutes. In eggs treated wih cold, the time required for the re-establishment of the spindle is not affected by the duration of the cold treatment, even up to several hours.

During the reconstitution, the spindle goes through a regular series of morphological changes. First the small spindle migrates rapidly to the cell periphery, then moves slowly away from the surface again as the retardation and length of the spindle increase. At this stage, continuous fibers show strong birefringence, but after the retardation has reached a maximum, chromosomal fibers replace the continuous fibers. The retardation of the individual chromosomal fibers fluctuates for some time until finally the chromosomes are regularly arranged on the metaphase plate. Once the spindle is completed, the birefringence of the spindle is fairly stable for a given temperature.

The equilibrium retardation is a function of temperature, and is greater the higher the temperature up to a maximum at 28–30° C. At the same time, the retardation of the spindle increases proportionately with spindle length. Since the spindle tends to be longer at higher, and shorter at lower temperature, it would appear as if the temperature dependence of the retardation were correlated with the change in length. However, it can be shown that even at equivalent lengths, the retardation of the same spindle is greater at a higher temperature.

Studies on the distribution of vitamin B_{12}. CHARLES A. NICHOL.

Vitamin B_{12} is required by many animal species; in man, lack of this vitamin causes pernicious anemia and in many mammalian and avian species an inadequate supply of vitamin B_{12} results in depression of growth. However, yeast and the tissues of higher plants are apparently devoid of vitamin B_{12}. Supply of this vitamin is dependent upon the synthetic ability of many microorganisms; other microorganisms requiring vitamin B_{12} are useful in sensitive assay procedures. In this study, measurement and identification of vitamin B_{12} were made by means of a microbiological assay (*Lactobacillus leichmannii* ATCC 7830) and paper chromatography. The present investigation was directed along two lines: (1) *Distribution in tissues of invertebrates.* Our knowledge of the distribution of vitamin B_{12} in invertebrate species is very limited. Several reports have indicated the presence of vitamin B_{12} in certain molluscs and annelids but in each case, assay of the whole organism prevented relationship of the activity to the metabolic processes of particular tissues. In this study, extracts of tissues from representatives of invertebrate phyla were prepared for analysis. With few exceptions, clearly defined tissues were separated for treatment. (2) *Distribution in relation to function.* Vitamin B_{12} has been implicated broadly in the metabolism of nitrogen and in the formation of methyl groups. In the marine elasmobranchs a very large proportion of the waste nitrogen is detoxified and excreted as trimethylamine oxide. Since this product can be reduced to a volatile amine and readily isolated, study of the formation of trimethylamine oxide should be advantageous to the detailed study of the function of vitamin B_{12}. As a preliminary step in such a study, tissues of several elasmobranchs (smooth dogfish, ray and torpedo) were prepared for analysis for vitamin B_{12}.

Glycolytic activity in the swim bladder gland. C. F. STRITTMATTER, E. G. BALL AND O. COOPER.

The present study is a contribution toward clarifying the possible role of the so-called red gland of fish swim bladders in the selective release of oxygen into the swim bladder. It has been suggested that the oxygen is released from oxyhemoglobin of the blood as the result of the metabolic production of an acid by this gland.

The acid production of swim bladder glands from the common scup was followed *in vitro* by manometric determination of carbon dioxide release in a 0.225 M sodium chloride-0.025 M sodium bicarbonate medium at 30° C. with an atmosphere of 5% CO_2-95% N_2. Usually each gland was bisected and one-half run as a control. Addition of glucose (75 mg. % final concentration) increased acid production ten- to twenty-fold, to about 6 micromols per 100 mg. fresh gland per hour. Mannose, fructose and galactose in this same concentration produced 87.5, 30.5 and 14.2%, respectively, of the activity observed with glucose. The activity observed with glucose as substrate was inhibited about 75% on replacement of the sodium in the medium by potassium. Addition of phosphate or of magnesium or calcium ions to the limit of solubility in the medium produced no appreciable effect on acid production. Changing the pH of the medium from 7.3 to 7.0 caused a 50% drop in acid production; a rise to pH 7.6 produced a 25% increase.

The acidic product of glycolysis was identified as lactic acid. Under anaerobic conditions and with an initial added glucose concentration of 75 mg. %, lactic acid formation accounted, on the average, for 75.5% of the glucose utilized and 90.3% of the acid production. These percentages were somewhat lower when no glucose was added and slightly higher with 150 mg. % glucose. The same pattern of metabolism was observed under aerobic conditions (5% CO_2-95% O_2), both with respect to amount of glucose utilized and the percentages of utilization and acid production accounted for by lactic acid.

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THE ACTION OF DN-ASE ON CELL NUCLEI[1]

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DNase (desoxyribonuclease) has been used as a cytochemical tool for some time because the unique localization of its substrate, DNA (desoxyribonucleic acid), calls for an investigation of its role in the structural organization of the nucleus. Mazia and Jaeger (1939) and Mazia (1941), using relatively impure nuclease preparations, demonstrated that the enzyme removed DNA from Drosophila and Chironomus chromosomes to the point of Feulgen-negativity without destroying the fibrous structure of the chromosomes. These basic observations have since been confirmed by Frolova (1941, 1944) and by Kaufmann and his co-workers, using highly purified enzyme preparations (Kaufmann, 1949; Kaufmann, Gay and McDonald, 1950). These workers have carefully examined the problems and possible errors of the cytochemical use of enzymes.

The fact of removal of DNA from chromosomes by DNase is unquestioned. Also unquestioned is the fact that the chromosome and nuclear structure is preserved after DNase action. The questions that the cytochemical experiments raise and cannot answer are: (1) is all the DNA homogeneous with respect to its removal by the enzyme, and (2) are any other constituents removed along with the DNA?

Such quantitative data may be obtained by analyzing the effects of DNase on isolated cell nuclei. Large amounts of material and straightforward chemical techniques may be used. An examination of how DNase can remove DNA from the nucleus, presumably by splitting inter-nucleotide bonds, can be expected to throw some light on the nature of the association between protein and nucleic acid in the intact nucleus.

MATERIALS AND METHODS

Mammalian nuclei were isolated by the Mirsky-Pollister techniques using dilute citric acid (1946b). The nuclei were washed and suspended in buffer solution, either Sorenson's phosphate buffer or Michealis' veronal mixture.

Invertebrate sperm from various marine species were collected and washed with filtered sea water. The sperm were then shaken with dilute citric acid and washed and suspended in buffer solution.

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[1] Part of this paper is taken from a dissertation presented to the Faculty of the Graduate School of the University of Missouri, Feb., 1951, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Support from the American Cancer Society, recommended by the Committee on Growth, National Research Council, through their grant to Dr. Daniel Mazia, is gratefully acknowledged.

[2] Atomic Energy Commission Predoctoral Fellow, 1949–50. Part of the data was collected during the tenure of this Fellowship.

Carp sperm were collected from ripe fish and washed in a saline mixture (Mirsky and Pollister, 1946a). Turtle erythrocytes were washed in 0.18 *M* NaCl, hemolyzed in distilled water, gently blended in the Waring Blendor with dilute citric acid and washed until as free as possible from cell membranes. The reddish brown color of the nuclei indicated considerable contamination with hemoglobin.

Desoxyribonucleic acid was determined by the Dische diphenylamine method. The use of the Dische reaction directly on nuclear suspensions was justified by independent measurements using the Schneider technique for the extraction of nucleic acids from proteins prior to the diphenylamine test. When clear supernatants were measured, the Dische reaction was checked by independent measurements using UV absorption at 260 millimicrons in the Beckman spectrophotometer.

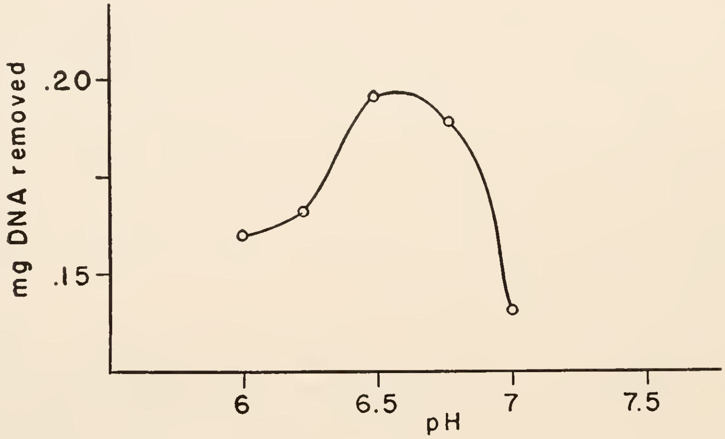

FIGURE 1. The pH optimum for removal of DNA from thymocyte nuclei by DNase. The points represent the amount of DNA removed in the first hour of digestion by the enzyme.

Amino nitrogen was measured by the Van Slyke manometric technique (1929). Hydrolysis of the sample for analysis was accomplished in 6 N sulfuric acid for 24 hours.

PROCEDURE

The nuclei were suspended in the buffer solution at pH 6.5. This was determined to be the optimum pH for the removal of DNA from the nucleus by DNase (Fig. 1). $MgCl_2$ was added as an activator to a final concentration of 0.01 *M*. Merthiolate (obtained through the courtesy of Eli Lilly Co.) was used in a final concentration of 1 : 10,000 to inhibit the growth of micro-organisms. Viscometric measurements of the activity of DNase in depolymerizing DNA in the presence of varying amounts of merthiolate were made by J. Neff (unpublished). No effect on DNase could be observed at the concentration of 1 : 10,000 used to inhibit bacterial growth. Crystalline DNase from the Worthington Biochemical Co. was then added. The mixture was incubated at constant temperature, 29.2° C. Samples were taken at intervals and centrifuged at about 4000 RCF. The clear supernatant, free of nuclei, was analyzed for DNA. Control experiments were identical except that no enzyme was added.

RESULTS

Release of DNA. As the digestion of the nuclei with DNase proceeds, DNA is released into the medium. The time course of the reaction is shown in Figure 2. The curve, representative in general of all experiments, describes the digestion of the sperm nuclei of the surf-clam, Mactra (Spisula). The most striking feature of the reaction is that not all the DNA can be removed from the nuclei even after prolonged digestion.

This effect, the failure of the DNase to remove all the DNA from the nuclei, has been found to occur in all forms tested. Table I shows the results of the enzymatic digestion of isolated thymocyte nuclei at various ratios of enzyme to substrate. The lowest value of 65.6 per cent DNA released is associated with the lowest enzyme-substrate ratio. The time curve of this particular reaction shows that the plateau had not been reached even after 35 hours of digestion. Table II summarizes the results of the digestion of nuclei from a wide variety of organisms. In all cases the amount of DNA left behind, *i.e.,* resistant to removal by DNase, is closely reproducible. The results of duplicate experiments on the same batch of nuclei ranged between two to three per cent. Variation among different batches of nuclei did not exceed this range. The differences in the amount of resistant DNA are associated with species characteristic differences and not to methodological variation.

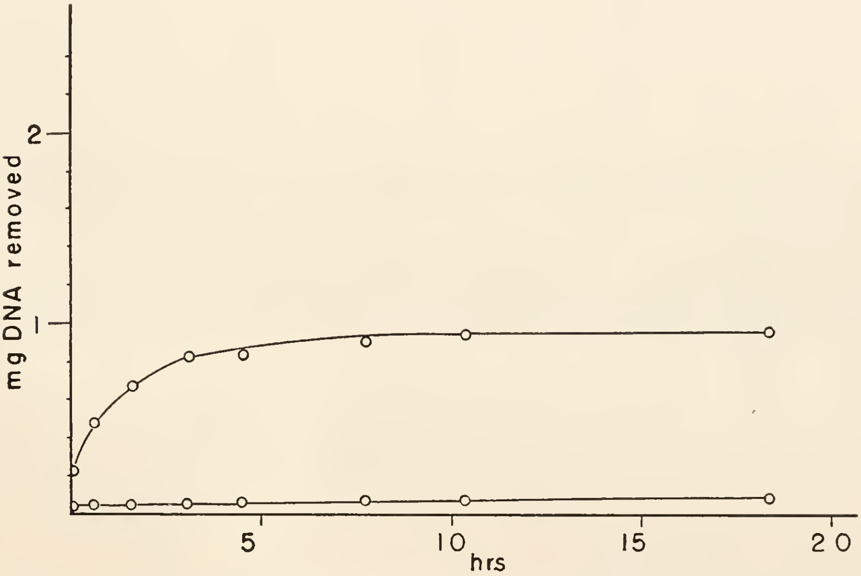

FIGURE 2. Time course of the removal of DNA from Mactra sperm nuclei by DNase. Sperm suspension in 0.05 M phosphate buffer, pH 6.5, 0.01 M with respect to $MgCl_2$. Enzyme conc. 4.0 microgm./ml. Total DNA = 2.5 mg./ml.

That the DNA remaining with the nuclei is truly resistant to digestion and removal can be demonstrated by the following observations. (1) Addition of fresh enzyme and/or activator to the reaction mixture does not result in additional release of DNA. (2) Washing the nuclei in fresh buffer solution does not remove any more DNA. (3) Washing and re-suspending the nuclei in fresh buffer, adding fresh enzyme and $MgCl_2$ does not result in further digestion. Such intensive checks were made in particular on thymocyte nuclei and on Mactra sperm nuclei. Table III shows the results of the re-digestion of Mactra sperm nuclei with fresh enzyme.

TABLE I

Release of DNA from beef thymocyte nuclei by DNase

Batch	Enzyme conc. mg./ml.	DNA in suspension mg./ml.	DNA released	
			Amount	Per cent
1	0.100	1.56	1.20	77.0
2	0.028	1.64	1.27	77.5
3	0.013	0.70	0.46	65.6
4	0.050	0.91	0.71	78.4
4	0.050	0.91	0.69	75.8

Release of protein. On the basis of a cytological approach to the problem of the mode of action of DNase in removing DNA from Chironomus salivary-gland chromosomes, Barton (Barton and Mazia, 1949) proposed that the depolymerization of the DNA of the chromosomes led to the formation of low-molecular weight nucleoprotein complexes that were soluble at the ionic strength of the buffer solutions. Such a concept was in line with the behavior of artificial low-molecular weight DNA and basic protein complexes (Barton, 1948). Attempts were therefore made to determine the amount of protein released along with the DNA. On the basis

TABLE II

Amount of DNA removable by DNase from the nuclei of various animals and tissues

Animal	DNA removable, %
Asterias, sperm	85%
Pecten, sperm	89
Mytilus, sperm	84
Arbacia, sperm	74
**Strongylocentrotus purpuratus*, sperm	80
**Strongylocentrotus franciscanus*, sperm	80
Mactra, sperm	40
Carp, sperm	82
Turtle, RBC (Caretta)	80
Rat, liver	70
Steer, thymus lymphocytes	77

* The data on Strongylocentrotus were kindly collected by Dr. Daniel Mazia, U. of California.

TABLE III

Re-digestion of Mactra sperm nuclei. After removal of 40% of DNA sperm re-suspended in 0.05 M PO_4 buffer, pH 6.5, 0.01 M $MgCl_2$. DNase conc. = 4 microgm./ml. Total DNA = 2.4 mg./ml.

Elapsed time	With enzyme DNA removed mg./ml.	Without enzyme DNA removed mg./ml.
1′	0.05	0.03
54′	0.05	0.03
7 hr. 46′	0.05	0.03

of Mirsky's data on isolated thymus chromosomes, the minimum amount of basic protein in the lymphocyte nucleus is about two-thirds the amount of DNA. The minimum amount of protein expected to accompany the release of DNA may then be calculated. The manometric method of Van Slyke has the requisite sensitivity and accuracy to measure this minimum amount of protein. Table IV compares the results of the analysis with the calculated amounts. It is obvious that no protein is released with the DNA. DNase removes only DNA and only part of that. The figures are corrected for the DNA nitrogen which is affected by the hydrolysis and which reacts with the nitrous acid in the Van Slyke method. The correction for 24 hours hydrolysis and a four-minute reaction in the manometric chamber amounts to about ⅕ of the purine and pyrimidine nitrogen—0.03 mg. N/mg. DNA.

Visible changes. During the process of digestion, the nuclei were examined under the phase contrast microscope. Two changes could be noted in the case of beef thymocytes. First, there was a pronounced loss in size. Accompanying the loss in size was a progressive wrinkling and deformation. Second, the contrast of the nuclei was gradually decreased. Undigested nuclei isolated by the citric acid method present a microscopic picture of sharp contrast. As digestion proceeds, the contrast becomes flat. The integrity of the nucleus is not lost, however. There is no tendency for the nucleus to dissolve or rupture.

Pretreatment of nuclei. When using mammalian tissues, the isolation procedure of necessity introduces a number of preliminary steps to the digestion experiments. These steps might be expected to affect the nucleic acid of the nuclei. Washing with citric acid is particularly suspect in this regard. By the use of invertebrate

TABLE IV

Determination of release of amino nitrogen accompanying removal of DNA from thymocyte nuclei by DNase

Digestion time hours	Amino N found* mg./ml.	Amino N expected** mg./ml.
0	+0.0068	0.007
3.3	−0.0050	0.055
13	+0.0087	0.075

* Difference between the blank and test analyses.

** Calculated on the basis 0.66 mg. protein per mg. DNA. Amino N is approximately 16 per cent of the protein.

sperm, it was thought possible to test the action of the citric acid. Sperm from Asterias, Mactra and Arbacia were washed in sea water and then suspended in the digestion mixture, as described above in the section on procedures, without any other pre-treatment. Use of both phosphate and veronal buffers in the digestion mixtures quickly resulted in the death of the sperm as indicated by the loss of motility. However, DNase was now completely ineffective in removing any of the DNA of the sperm. Treatment with dilute citric acid allowed DNase to remove part of the DNA. Duration of the treatment with citric acid did not appear to be significant. Nuclei washed once in one per cent citric, total exposure about 15 minutes, were as susceptible to DNase as were nuclei stored for several days in the citric acid.

Removal of the resistant fraction. In an attempt to determine why the cytochemical digestion studies had not disclosed the existence of so large an enzymatically resistant fraction of DNA, several techniques, inherent in the cytochemical method, were tested for their ability to remove the resistant fraction. Mactra sperm nuclei, previously subjected to digestion with DNase until 40 per cent of the DNA had been removed, were collected and fixed in Carnoy's fixative for 20 minutes. The fraction is stable to Carnoy's fixative. The nuclei were then suspended in 1 N HCl and heated to 60°–70° C. as in the Feulgen reaction. Three-minute hydrolysis removed a small amount of the DNA. Twenty-five minute hydrolysis removed all the DNA in the nuclei. Mazia and Jaeger (1939) had noted that the cytoplasm of the salivary gland cells gave a positive Feulgen reaction after treatment of the cells with DNase. It seems probable that the acid hydrolysis plus the sulfurous acid of the basic-fuchsin reagent in the Feulgen reaction caused the loss of the enzyme-resistant fraction.

Treatment of the nuclei so as to remove the basic protein renders them susceptible to complete digestion by DNase. Previously digested Mactra sperm nuclei containing 60 per cent of their original DNA were extracted for one-half hour with 0.2 N HCl. This treatment removes some or all of the basic protein of the nucleus. The nuclei were then washed and suspended in phosphate buffer, pH 6.6, $MgCl_2$ and 4 microgm./ml. of DNase added. The DNase can now remove all the remaining DNA in 8 hours. Extraction with buffer alone removed one-seventh of the DNA after 12 hours. These observations demonstrate that the resistant DNA fraction is only resistant to removal from the intact nucleus and is not intrinsically immune to hydrolysis by DNase.

Treatment of the partially digested nuclei with 2 *M* NaCl will also remove the remaining DNA along with protein. The extracted complex does not form a true solution and hence is not viscous. Preliminary studies of this complex were reported in 1951 (Barton).

Discussion

The most important question arising from this work is why all the DNA does not come out when subjected to digestion by DNase. There is, of course, no *a priori* reason why breaking internucleotide bonds should result in the removal of the hydrolyzed fragments. The effectiveness or lack of effectiveness of DNase in removing DNA must be related to the nature of the linkage between DNA and the protein components of the nucleus or to the spatial orientation of the DNA in the nucleus.

Various hypotheses for the failure of DNase to remove all the DNA may be suggested. (1) The enzyme has been inhibited by products of the reaction. This proposal may be rejected on the basis of the redigestion experiments with fresh enzyme. Washing of the nuclei should have removed any inhibitor. (2) All the DNA may have been digested, but the fragments have complexed with or adsorbed on the proteins of the nucleus. In such a case, an equilibrium should exist beween DNA complexed and DNA in solution. Washing should shift the equilibrium and remove complexed DNA fragments. This is not observed.

Can the DNA remaining with the nuclei be identified with any DNA fraction previously reported in the literature? Zamenhof and Chargaff (1949) reported the discovery of an enzymatically resistant fraction of beef thymus DNA. Similar results have been reported by Overend and Webb (1950). It is clear that the fraction described in this paper is enzymatically resistant to removal, but the amount of the fraction is considerably larger than the "core" reported by Chargaff and by Overend. The core reported in thymus DNA amounted to no more than 7 per cent of the total DNA. Chargaff's analysis of the core showed that it contained a higher ratio of purines to pyrimidines than did the intact DNA. The remainder of the DNA, *i.e.*, the dialyzable portion of the enzymatic digest, showed a higher ratio of pyrimidines to purines than did intact DNA. In the experiments reported here on the removal of DNA from nuclei, analyses of the DNA released by the enzyme were made by UV absorption, which measures both purine and pyrimidine bases; these checked closely with analyses made by the diphenylamine reaction which gives a measure only of purine-containing nucleotides. Pending a quantitative analysis of the bases in the removable and resistant fractions, the conclusion is reached that the resistant fraction does not correspond to a core resistant to the enzyme by virtue of a high purine nucleotide composition. The ability of DNase to remove the fraction following extraction with 0.2 N HCl supports this conclusion.

Mirsky (1947) has reported that the isolated "chromosomes" of thymocyte nuclei contain two fractions of DNA, one removable with strong NaCl and a second residual fraction, only a few per cent of the total, associated with the residual chromosome. The amount of the discrepancy with an enzymatically resistant fraction of 23 per cent reported here is too great to suppose an identity between the two.

The evidence at present points to the conclusion that the fraction of DNA resistant to removal by DNase is a spatially separate component of the total DNA. It is not intrinsically resistant to DNase, but only so because of its location in the nucleus. Removal of basic protein by treatment with 0.2 N HCl allows the enzyme to act. The block to complete removal of the DNA by DNase may be interpreted in two ways. (1) First, the basic protein may take part in an actual masking of the substrate. Thus, one portion of the DNA, the easily removable one, would be "exterior" to the remainder of the nucleoprotein complex; the second portion of the DNA, the resistant one, would be "interior," surrounded by the basic protein. (2) The second DNA component may be bound to protein of the nucleus in such a manner as to be immune to attack by DNase. Treatment with 0.2 N HCl would not only extract the basic protein but would dissociate or alter the complex so that the DNase could now act. Preliminary studies of the resistant DNA complex, removed by extraction with strong NaCl solutions as described above, indicate that the nucleoprotein does not behave in a typical manner. In particular, it is not dissociated into DNA and protein by media of high ionic strength.

A choice between these possibilities must await detailed examination of the resistant fraction.

No explanation can be offered for the variation of the amount of resistant DNA among the various species studied except to point out that the amounts fall into two classes: one centering around 80 per cent, the other (Mactra) at 40 per cent. One feature of the chemistry of the nuclei of the species that also separates them into the same two classes is the nature of the basic protein contained in the nuclei. In all the species except Mactra, the basic protein is of the histone type containing approximately 20–25 per cent basic amino acids. Mactra, on the other hand, contains in its sperm nucleus a basic protein more closely resembling protamine in its solubility properties and would thus be expected to have a considerably higher proportion of basic amino acids (Barton, 1951).

The action of dilute citric acid in the pretreatment of the nuclei for digestion is not clear. The citric acid may affect the nuclei in at least two ways. (1) It may complex with calcium of the nuclei, thus altering the state of DNA and protein within the nuclei. (2) It may alter the nuclear membrane profoundly. A "toughening" effect of dilute acids on nuclear membranes was first observed by Crossmon (1937). Further study with other calcium complexing agents and with other means of killing are in progress.

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Summary and Conclusions

1. The digestion of isolated nuclei by DNase has led to the following conclusions: (a) not all the DNA can be removed by the enzyme; (b) no protein is released with the DNA; (c) treatment of the residue with 0.2 N HCl removes the basic protein; it does not remove the DNA, but renders the resistant fraction susceptible to digestion with DNase.

2. The resistant fraction of the DNA cannot be correlated satisfactorily with any previously reported DNA fractions. The resistance to removal can be interpreted as due to the spatial orientation of the DNA within the nucleus or to the unique mode of combination with protein of the nucleus.

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THE AGGLUTINATION OF FISH ERYTHROCYTES BY NORMAL HUMAN SERA

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The discovery that normal human sera strongly agglutinate the erythrocytes of some species of fish led the authors to investigate this phenomenon systematically. This study represents part of a general program (Cushing, 1952a, 1952b) intended to develop serological techniques that might be useful in making racial and evolutionary studies on fish similar to those being made on man (Race and Sanger, 1950) and such other animals as doves (Irwin, 1947) and cattle (Stormont, Owen and Irwin, 1950).

Although Nuttall (1904) included fish in his classical work on the serological differentiation of animal bloods, relatively little has since been done with the serology of this group of vertebrates. The references known to these authors are listed in the bibliography or may be found by consulting the papers given. These include work by the following: Streng, 1938; Cushing, 1942, 1945a, 1945b; Gemeroy, 1943; Jonsson, 1944; Tyler and Metz, 1945; Tyler, 1946; and Grubb, 1949.

In addition, attention is called to the work of Cotterman (1944) who used normal human serum to demonstrate differences in mice of the genus *Peromyscus* and to that of Tamm (1952) demonstrating species differences in the viral agglutination of fish erythrocytes.

In order to establish a point of departure for comparative study, two species of marine fish were selected, the shiner sea-perch, *Cymatogaster aggregata* (Gibbons), a member of the viviparous perches (Embiotocidae), and the kingfish, *Genyonemus lineatus* (Ayres), a croaker (Sciaenidae). These were chosen primarily because their red blood cells were strongly agglutinated by human sera and because of the ease with which they could be captured locally and kept alive in the laboratory.

MATERIALS AND METHODS

The physiological saline used was prepared by diluting filtered sea water with an equal volume of distilled water. This gave a final concentration of approximately 1.5% sodium chloride. The use of sea water avoided the possibility of hemolysis induced in the cells of some species of fish by some artificially prepared sodium chloride solutions (Ball, 1933). The agglutination of human erythrocytes was not affected by the use of this saline.

Fish erythrocytes were obtained by heart puncture. Blood was drawn into syringes containing an approximately equal volume of heparin solution. This solution had a concentration of 50 mg. of heparin for every 10 ml. of physiological saline. This preparation was found to be satisfactory for most of our work, but further research on the usefulness of other concentrations of heparin and on other kinds of anti-coagulants is desirable.

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Erythrocytes were washed free of serum proteins in physiological saline and finally suspended in a concentration approximating two per cent cells.

The human sera used in these studies were obtained either from local individuals, in which case their complement was heat-inactivated before use, or in the case of blood typing sera, from the Cutter Laboratories, Berkeley. The particular sera used in an experiment are referred to in the legends of the tables presenting the data for that experiment. Sera were kept frozen when not being used, inasmuch as repeated freezing and thawing were not observed to have any effect upon the agglutinins being studied.

Slide agglutination tests were used to determine the presence or absence of the various antigens and antibodies studied. These tests were made at room temperature by placing single drops of the appropriate cell suspensions and sera together and mixing these by hand rotation of the slide. Readings were made by eye at the end of 15 minutes without the aid of a microscope and checked at 30 minutes. Drying was prevented by placing Petri dish covers over the slides.

Absorptions of sera by erythrocytes were made by placing 0.1 ml. of packed cells in small test tubes with 1.0 ml. of serum and either rotating these mixtures at a nearly horizontal angle by attaching the tubes to a disc turned on a stirring motor, or by placing the tubes in a horizontal position and shaking them through their long axes on a Yankee-Kahn Shaker. In this later procedure, a small glass bead was added to each tube. The time of absorption varied in individual cases from 5 to 20 minutes. In general it was found that a five-minute absorption on the shaker was sufficient to obtain suitable preparations.

These preparations were obtained with a view of decreasing the titers of the antibodies being absorbed relative to those not being absorbed to a point where the slide test gave negative readings of the absorbed antibody while showing that the titers of unabsorbed antibody remained the same. While the slide test was entirely adequate for the studies reported here, it has been suggested that the test tube method (Schiff and Boyd, 1942; pp. 31–33), enhanced by centrifugation, may prove to be a faster and more sensitive test for future work.

Care was taken to insure that all the sera used were sterile and therefore did not contain agglutinins produced by bacteria.

RESULTS

The antigens described below represent the minimum number of antigenic differences that can be detected between *Cymatogaster* and *Genyonemus* cells. The possibility exists that a single antigenic entity may be resolved into two or more components with the discovery of suitable antibodies. The F-3 and F-4 antigenic components described below were thus resolved.

The results of studies of the sera of several individuals, which were collected at random, indicate that the titers of red cell agglutinins for fish and humans vary independently. The indicated titers (Table I) should be taken as reasonable approximations only, inasmuch as it is possible that individual variations in red cells might influence the relative strength of the reactions to some extent (Wiener, 1943; p. 20). The agglutinins involved are stable to heating at 56° C. for 25 minutes. That dif-

TABLE I

Agglutination of fish and human erythrocytes by normal human sera

Serum from human type	Cells from	Serum dilutions				
		1/4	1/8	1/16	1/32	1/64
AB	*Cymatogaster*	3+	+	±	0	0
	Genyonemus	0	0	0	0	0
AB	*C.*	+	±	0	0	0
	G.	0	0	0	0	0
A	*C.*	2+	+	0	0	0
	G.	±	0	0	0	0
	B (Human)	±	0	0	0	0
A	*C.*	4+	4+	3+	2+	+
	G.	±	0	0	0	0
	B H.	±	±	0	0	0
B	*C.*	4+	3+	3+	±	0
	G.	2+	±	0	0	0
	A H.	+	0	0	0	0
B	*C.*	2+	+	0	0	0
	G.	0	0	0	0	0
	A H.	2+	±	0	0	0
O	*C.*	4+	4+	3+	2+	0
	G.	3+	+	0	0	0
	A H.	2+	+	±	0	0
	B H.	3+	2+	+	0	0
O	*C.*	3+	3+	+	±	0
	G.	+	0	0	0	0
	A H.	+	±	0	0	0
	B H.	+	+	±	0	0
O	*C.*	4+	3+	3+	2+	2+
	G.	2+	±	0	0	0
	A H.	3+	3+	+	0	0
	B H.	3+	+	+	0	0

The reactions of several human sera with fish and human erythrocytes are shown. All sera were from a single collection, twenty-four hours old, and were heat-inactivated at 56° C. for 25 minutes before using. Agglutination tests were run as described in the text. Degree of agglutination, estimated visually in terms of the number of cells agglutinated, is described as follows: 4+ = essentially all; 3+ = three-fourths; 2+ = one-half; + = one-fourth; ± = definite agglutination but less than one-fourth.

ferent agglutinins from the anti-A anti-B agglutinins may be involved in the reactions is suggested by the fact that the fish and the human red cell titers vary independently. Further evidence for this conclusion is obtained by the absorptions indicated in Table II. It can be seen from this table that since *Cymatogaster* cell absorption of anti-A plus anti-B sera fails to remove either anti-A or anti-B from the pooled sera, *Cymatogaster* cells must possess at least one antigen distinct from the A and B antigens on human cells. This is at once apparent when the sera of type AB individuals are studied since these individuals lack detectable anti-A or anti-B bodies in their sera but show titers against fish red cells.

Table II

Differentiation of an antigen on Cymatogaster erythrocytes

Unabsorbed sera	Cells			Sera absorbed with these cells	Cells		
	Human A	Human B	*Cymatogaster*		Human A	Human B	*Cymatogaster*
Anti-A	4+	0	4+	Human A	0	0	4+
Anti-B	0	4+	4+	Human B	0	0	4+
Anti-A+B	4+	4+	4+	*Cymatogaster*	4+	4+	0

The antisera were commercial preparations derived from pooled human sera. These were used in a final dilution of 1 in 2 from that of the original preparation as received in this laboratory. The anti-A+B serum was prepared by mixing equal volumes of the anti-A and anti-B and was used without further dilution.

The reactions of *Genyonemus* cells with human typing serum are more complex (Table III). *Genyonemus* cells carry an antigenic component that is different from that of *Cymatogaster* for it resembles the human B antigen in its reactions with anti-B serum. Further evidence for this point is the fact that *Genyonemus* cells are much more rapidly and strongly agglutinated by anti-B serum than by anti-A serum.

Table III

Demonstration of an antigen on Genyonemus cells that is serologically related to the human B antigen

Cells	Unabsorbed serum			Absorbed with *Genyonemus* cells			Absorbed with B cells		
	1/4	1/8	1/16	1/4	1/8	1/16	1/4	1/8	1/16
Human B	4+	3+	+	+	0	0	0	0	0
Cymatogaster	4+	3+	+	4+	3+	+	4+	3+	+
Genyonemus	3+	2+	0	0	0	0	0	0	0

The serum used was obtained from a type A human and was selected because it not only contained human anti-B and *Genyonemus* red cell agglutinins, but *Cymatogaster* red cell agglutinins as well.

Genyonemus cells react as strongly with anti-A typing serum after absorption of the anti-A antibodies as before (Table IV). The *Genyonemus* cells have at least one antigen other than the B antigen on their cells. This antigen is not related to human A. This point is confirmed by the occurrence of a human AB serum that agglutinates *Genyonemus* and *Cymatogaster* cells, but not human cells of type A or B (Table V).

It is therefore concluded that *Genyonemus* erythrocytes possess one antigen very similar to the human B antigen, and a second antigen distinct from either the human A or B antigen. *Cymatogaster* can be shown to possess one antigen distinct from the human A or B antigens.

For convenience in further discussion the two *Genyonemus* antigenic components will be numbered F-1 (the B-like antigen) and F-2 (the not-B antigen). The *Cymatogaster* antigen will be numbered F-3. That it is distinct from F-1 and F-2 will be shown below. The letter F is used here to denote that these are antigens found on fish cells, and to give distinctiveness to the numbers used.

TABLE IV

Differentiation of Genyonemus and human A antigens

Cells	Dilutions of unabsorbed anti-A			Dilutions of anti-A absorbed with human A cells		
	1/1	1/2	1/4	1/1	1/2	1/4
Human A	4+	4+	3+	0	0	0
Cymatogaster	4+	3+	±	4+	3+	±
Genyonemus	2+	±	0	2+	±	0

The antiserum used was a commercial preparation of pooled human sera and was diluted after absorption.

The differentiation of F-2 and F-3 is based upon several facts. First, the anti-B serum absorbed with *Genyonemus* cells still retained an unimpaired ability to agglutinate *Cymatogaster* cells (Table III) which they would not have done had F-2 and F-3 been alike antigenically. Second, the absorption with *Genyonemus* cells of the AB serum capable of agglutinating both *Genyonemus* and *Cymatogaster* cells left the *Cymatogaster* agglutinins intact (Table V).

TABLE V

Differentiation of an antigen on Genyonemus cells

Cells	Dilutions of unabsorbed serum				Dilutions of serum absorbed with *Genyonemus* cells			
	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16
Cymatogaster	4+	4+	+	0	4+	4+	+	0
Genyonemus	4+	+	0	0	0	0	0	0

The antiserum used was from an AB individual selected because it agglutinated both *Cymatogaster* and *Genyonemus* cells, but failed to agglutinate human A, B or sheep red cells. The serum was diluted after absorption.

Additional evidence shows that *Cymatogaster* and *Genyonemus* cells each have at least one antigen peculiar to themselves, although not necessarily F-2 and F-3. A rabbit anti-*Cymatogaster* serum that agglutinated *Cymatogaster* cells in dilutions greater than 1 in 600 failed to agglutinate *Genyonemus* cells in any dilution. Also a rabbit anti-yellowfin tuna serum strongly agglutinated *Cymatogaster* cells and only weakly agglutinated *Genyonemus*. The reverse was true of an anti-skipjack serum that did not agglutinate human B cells.

Once distinguished, the F-3 (*Cymatogaster*) antigen could be further resolved into two separate antigenic components on the basis of the following observations. Sera of human AB individuals were found which agglutinated *Cymatogaster* but not sheep cells. The designation F-3 is retained for the antigen involved in this agglutination reaction.

In addition, anti-sheep cell sera were found to agglutinate strongly *Cymatogaster* cells but not those of *Genyonemus*. Absorption of rabbit anti-sheep cell serum with *Cymatogaster* cells left agglutinins for sheep cells only, but sheep cells removed both sheep and *Cymatogaster* agglutinins from this serum (unabsorbed sheep cell titer 1 in 1000, unabsorbed *Cymatogaster* titer 1 in 64). These reactions therefore reveal a second antigen on *Cymatogaster* cells, designated as F-4.

These observations, together with the fact that sheep cells are hemolysed by the action of heat-inactivated rabbit anti-*Cymatogaster* serum (titer of hemolysin = 1 in 40) in the presence of guinea pig complement, suggest that F-4 is one of the so-called Forssman antigens (Boyd, 1947).

So far, no individual antigenic variations have been detected within the two species. However, a persistent search for these was not made.

Discussion

We find that normal human sera contain at least three agglutinins that are specific for antigenic components on the erythrocytes of *Genyonemus* and *Cymatogaster*. One of these is the anti-B agglutinin, while the other two represent antibodies not related to the classical isoantibodies. Whether these antibodies are genetically determined, or are the result of immune responses to infection (Wiener, 1951), remains to be found. The three antibodies serve to identify two antigenic components on *Genyonemus* red cells, one of which is similar to the human B antigen, and one antigenic component on *Cymatogaster*.

In addition, rabbit anti-serum against sheep erythrocytes has been used to demonstrate a second antigenic component on *Cymatogaster* erythrocytes. This component resembles the Forssman antigens in its properties.

The term antigenic component is used to differentiate the two serologically distinct entities of each species for the reason that available evidence cannot differentiate between two alternative hypotheses: (1) that each serologically recognizable component of a given fish is actually a distinct and separate antigen; (2) that each serologically recognizable component of a given fish represents a difference in the reactions of two kinds of antibodies with a single antigen on the cells of that fish.

In other words, unless the antigenic components can be separated genetically (or otherwise) it is possible that *Cymatogaster* and *Genyonemus* may differ only in a minimum of two antigens rather than four. The problem involved is similar to that encountered in the study of some of the antigens in cattle (Stormont, Owen and Irwin, 1950), although the situation in cattle is, of course, much more complex, involving genetic as well as serological data.

No individual variations in the occurrence of these antigenic components have been noted in local populations of the two species of fish.

An extensive comparative study of the distribution in fishes of antigens reacting with human agglutinins and anti-sheep serum has been made and will be reported in another paper.

It is possible that the fish cell agglutinins discovered in human serum may be of clinical interest, as for example in cases of allergies to fish foods.

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SUMMARY

1. Normal human sera were found to agglutinate the erythrocytes of the shiner sea-perch (*Cymatogaster aggregata*) and of the kingfish (*Genyonemus lineatus*).

2. Agglutination was shown to occur by virtue of four different antigens, each species having two of these four in its erythrocytes.

3. Of these, *Genyonemus* had an antigen with a specificity closely resembling the human B substance (designated here as F-1) and a species-specific antigen (F-2) peculiar to itself.

4. *Cymatogaster* had a species-specific antigen (F-3) and an antigen with a specificity resembling that of the Forssman antigen (F-4).

5. No individual variations were found in either fish with respect to these antigens.

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PHOTOREACTIVATION IN COLPIDIUM COLPODA[1]

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Photoreactivation or reversal of the injurious effects of ultraviolet radiations (UV) by subsequent illumination with visible light has been observed in a variety of forms (for references, see Kelner, 1951 and Wells and Giese, 1950) but only one species of protozoan has been tested, namely *Paramecium aurelia* (Kimball and Gaither, 1951). Although considerable information has accumulated on photoreactivation and wave-length dependence of photoreactivating light, temperature dependence, photoreactivation time span etc. have been studied, the mechanism by which photoreversal occurs is still obscure. Additional data on forms other than those already studied may therefore be useful for this purpose. The present paper presents information of this kind on *Colpidium colpoda*, a ciliate protozoan. This form was chosen because it has a relatively high and regular division rate and is sufficiently large to be handled with ease and to be seen and counted with low powers of the dissecting microscope even with red light. Cultures were grown at 26° C. in 0.05% lettuce extract seeded the day before with *Pseudomonas ovalis,* in small tubes made from 0.4 mm. soft glass tubing, as described in detail elsewhere (Giese, 1945b).

METHODS

The UV radiation from a quartz mercury arc run at atmospheric pressure and at 200 volts, 2.2 amperes, was passed through a natural quartz monochromator to resolve the light. The desired wave-length was focused manually on a slit between two razor blades. The light passing through the slit was reflected onto a horizontal quartz cell by a right angle quartz prism. Visible light of various wave-lengths was obtained by passing through a monochromator radiations from a GE medium pressure mercury arc. In each case the intensity of the light was measured by a thermopile calibrated against standard lamps (U. S. Bureau of Standards).

After irradiation with UV the colpidia were always handled in darkness or in dim red light from darkroom safety lights tested spectroscopically to make sure that only red light was transmitted, since some defective red bulbs transmit parts of the entire spectrum. Observations and counts were made under low power (× 6.6) with a B. and L. dissecting microscope. The number of individuals in a tube was counted three times daily and averaged for a given series. From these data the division rate was determined as described in previous reports (Giese, 1939). The light used for illuminating the colpidia while counting them was enclosed in a black cover and the beam was passed through a water cell and a Corning filter #2412 which transmits mainly wave-lengths 7400–6150 Å (cut off at λ5950 Å).

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[1] Supported in part by funds made available by the Rockefeller Foundation.

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EXPERIMENTAL

The first experiments were designed to determine whether photoreactivation occurred. The colpidia were irradiated with a series of dosages of UV at λ2654 Å and then treated with blue light of λ4350 Å. It was apparent in the first experiments that photoreactivation of considerable degree had occurred. The nature of the experiments and the analysis of the data are illustrated in Figure 1. The dosages of λ2654 Å tried were 750, 1000, 1500 and 3000 ergs/mm². After the colpidia had been treated with a dosage of 3000 ergs/mm². at λ2654 Å, about 70% died and the remainder were greatly retarded in division. Yet in a sample of the same batch treated with a dosage of 113,000 ergs/mm². of blue light after the UV, all individuals survived and divided, the degree of retardation corresponding approximately to the amount found after somewhat less than 1000 ergs/mm². of the UV alone. Colpidia treated with UV plus visible in all cases act as though they had been given much lower dosages of UV alone (see Kelner, 1949, dose reduction principle). Colpidia were exposed to a dosage of 1000 ergs/mm². of UV in all subsequent experiments because it measurably retards division, yet recovery of normal division rate occurs within a reasonable time (several days). When larger dosages are used the experiments last too long a time; it is then possible that various factors other than irradiation become limiting. Thus the medium containing bacteria deteriorates as a growth medium for Colpidium in 6–8 days. While transfer from old medium to new can be performed it involves undesirable handling of the animals.

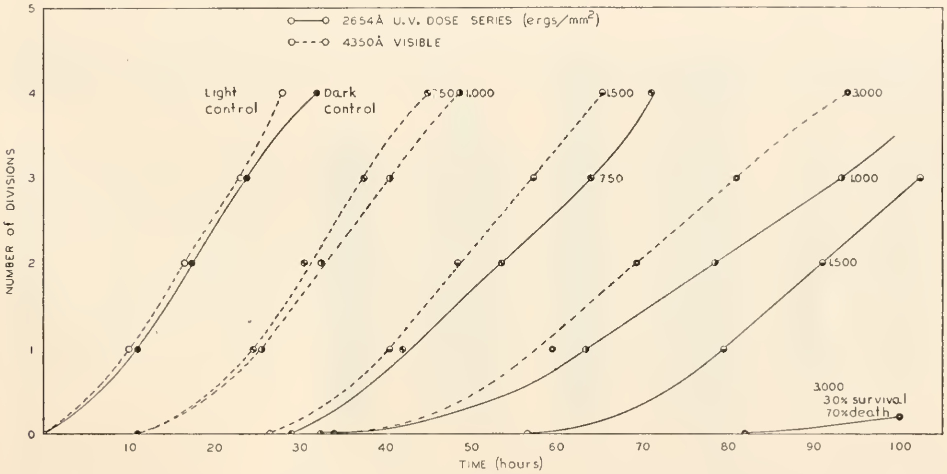

FIGURE 1. Effect upon division delay in Colpidium of various dosages of λ2654 Å with and without subsequent exposure to 56,900 ergs/mm². of blue light (4350 Å).

The second series of experiments concerned itself with the amount of blue light necessary for maximal photoreversal of retarded division of Colpidium after UV treatment. In Figure 2 are given data for a series of experiments in which colpidia were irradiated with a dosage of 1000 ergs/mm². at λ2654 Å and were then illuminated with blue light (λ4350 Å) of approximately the same intensity but of dif-

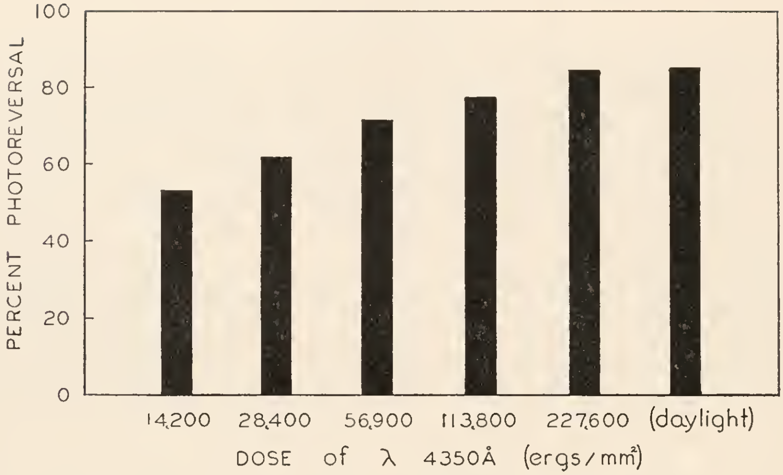

FIGURE 2. Effect upon division delay in Colpidium of various dosages of blue light (4350 Å) after a dosage of 1000 ergs/mm². of λ2654 Å. In one case the effect of daylight is compared to the effect of blue light.

ferent dosages. A progressively increasing degree of photoreversal occurs with increasing doses of blue light. However, even a 16-fold increase from the smallest to the largest tested increased the average photoreactivation only from 56% to 84%. Similar results on bacteriophage have been reported by Dulbecco (1950). The possibility that the mixture of visible wave-lengths as obtained in daylight might be more effective than blue light was tested in an experiment in which the UV-treated colpidia were exposed to indirect daylight for four hours on a bright day.

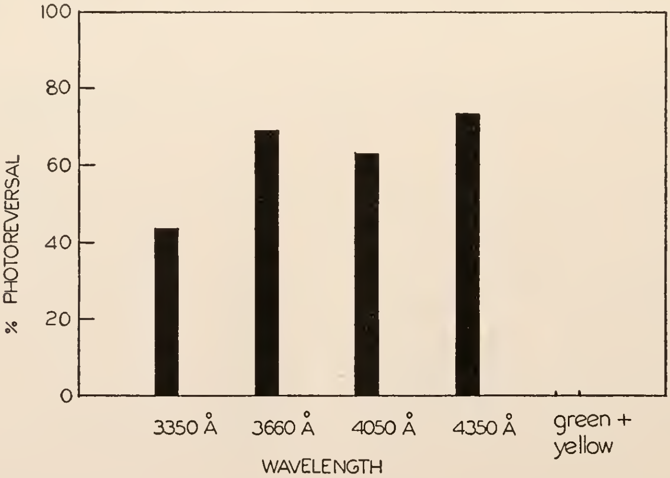

FIGURE 3. Effect upon division delay in Colpidium of a dosage of 56,900 ergs/mm². of various wave-lengths of long ultraviolet and visible light after a dosage of 1000 ergs/mm². of λ2654 Å.

The last bar in Figure 2 shows that reactivation after this large dosage of daylight is no greater than after 227, 600 ergs/mm^2. of blue light. While daylight might serve for photoreactivation studies, it is variable qualitatively and quantitatively; therefore a measured dosage of blue seemed preferable. While maximal photoreactivation with blue light might be desirable in some experiments, the time involved in giving a dosage of 227,600 ergs/mm^2. of blue light was too long to be practicable for most experiments; therefore a dosage of 56,900 ergs/mm^2. was settled upon as giving an adequate and satisfactory photoreversal, only 12% short of the maximal achievable.

To determine whether light other than blue might be more efficient for photoreversal in colpidia, the effect on photoreversal of several other wave-lengths (long ultraviolet: λ3350, 3660; violet, 4050; and yellow-green) available in the spectrum of the mercury arc was compared with blue. The data are summarized in Figure 3. In all cases colpidia were first exposed to 1000 ergs/mm^2. of UV of λ2654 Å usually followed by 56,900 ergs/mm^2. of the photoreactivating light. It will be observed that the long UV (3350 and 3660 Å), the violet (4050 Å), and the blue (4350 Å) are quite effective for photoreversal. On the other hand yellow-green has no effect or possibly a slightly injurious effect. Since blue occurs in high intensity in the mercury arc spectrum, it was used for reactivation in all succeeding experiments except in a few performed with the whole visible spectrum.

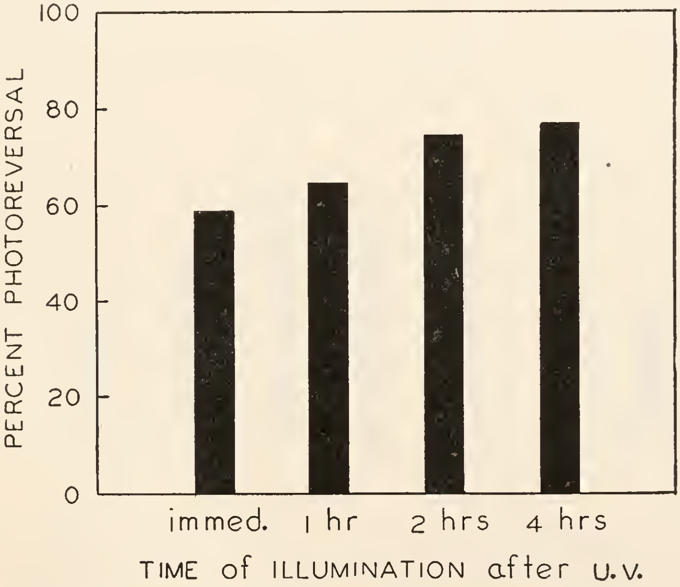

FIGURE 4. Effect of time lapse upon the degree of photoreactivation of division of Colpidium. The colpidia were given a dosage of 1000 ergs/mm^2. of λ2654 Å followed by a dose of 56,900 ergs/mm^2. of blue light (4350 Å) at the time specified.

In all cases a lapse of time occurred between the time of irradiation and the time of photoreversal. Since in studies on bacteria it was found by Kelner (1949) and others (*e.g.*, Novick and Szilard, 1949) that the time during which photoreversal can be achieved is relatively short at room temperature and in active cultures, the possibility arose that in the experiments reported here, the time between irradiation with UV and photoreactivation was too long. To determine whether any such loss in the capacity for photoreactivation occurred within a few hours following UV irradiation, illumination was performed at various times up to four hours after UV treatment. The amount of photoreactivation was observed to be even greater in some cases than if the blue light had been applied immediately after UV irradiation of colpidia [3] (Fig. 4). For the present series of experiments, it was considered quite adequate to perform the exposure to visible light within several hours of the irradiation with UV. Because of the possibility of an increase in photoreactivation with lapse of time after UV exposure, it was necessary to illuminate with blue light at about the same time lapse after irradiation in order that the experiments might be comparable.

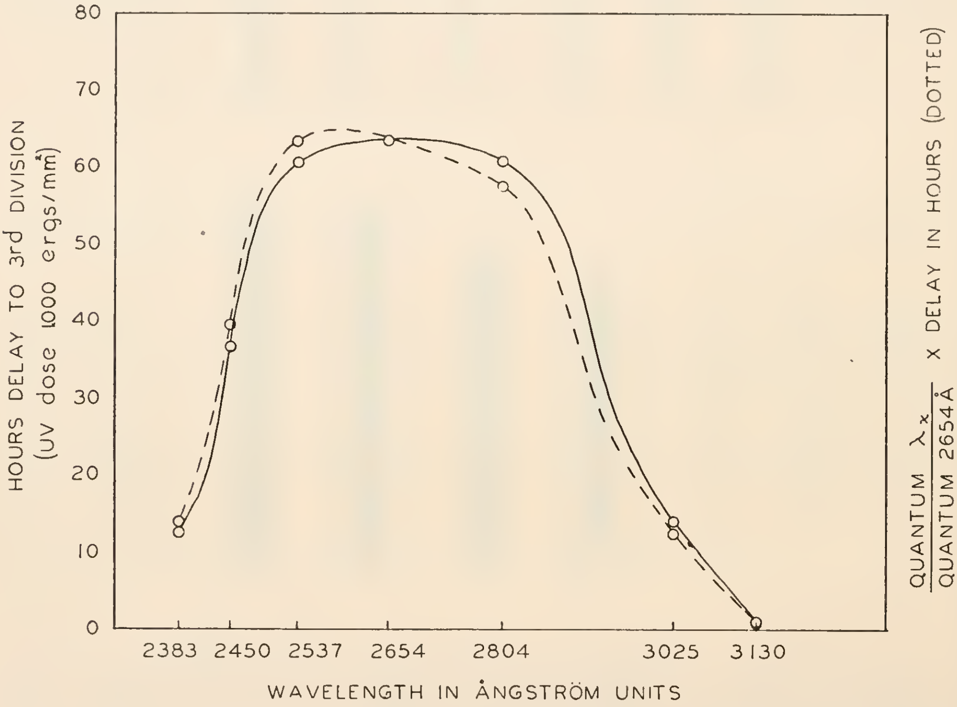

FIGURE 5. Relative efficiency of various wave-lengths of ultraviolet light upon division of Colpidium. The colpidia were irradiated with a dose of 1000 ergs/mm^2. at each of the wave-lengths. Comparison of relative efficiency on the basis of quanta is shown in the dotted curve.

The final set of experiments was planned as a comparative study of the degree of photoreactivation following exposure at various UV wave-lengths. In most cases a dosage of 56,900 ergs/mm^2. of blue light (4350 Å) was used for photoreactivation. Figure 5 shows the relative efficiency of a dosage of 1000 ergs/mm². of each of the various UV wave-lengths used in retarding the division of Colpidium. The effectiveness is compared in two ways: 1) on the basis of the energy incidence per/mm²., and 2) on the basis of the number of quanta per mm². The results give a crude action spectrum for the UV retardation of division. The most effective wave-lengths are 2537, 2654, and 2804 Å. Wave-lengths on the short end: 2450 and 2383 Å, and on the long end: 3025 and 3130 Å, are less effective in retarding division than are the intermediate wave-lengths. In Figure 6 is shown the degree of photoreactivation produced by exposing to a dosage of 56,900 ergs/mm². at λ4350 Å colpidia previously treated with 1000 ergs/mm². at each of the UV wave-lengths.[4] Illumination in all cases was performed shortly after irradiation.

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[3] Recent experiments carried out by C. L. Brandt (unpublished) indicate that the reactivation can be achieved for as long as 23 hours after UV treatment, probably until the first division after UV treatment but not thereafter.

Pretreatment of colpidia with blue light (dosage: 46,000 ergs/mm².) does not protect them significantly from subsequent exposure to UV (2654 Å, 1000 ergs/mm².). Colpidia so treated showed almost the same degree of retardation as those exposed to UV alone. Pretreatment with four hours of daylight before exposure to UV, however, produced some reduced sensitivity to UV applied thereafter. This phenomenon has been called *photodesensitization* by Weatherwax and Dobson (personal communication, 1951) who first observed it in *Escherichia coli* strain B. The decrease in sensitivity in Colpidium was only a small fraction of the photoreversal achieved by exposure to visible light after UV treatment.

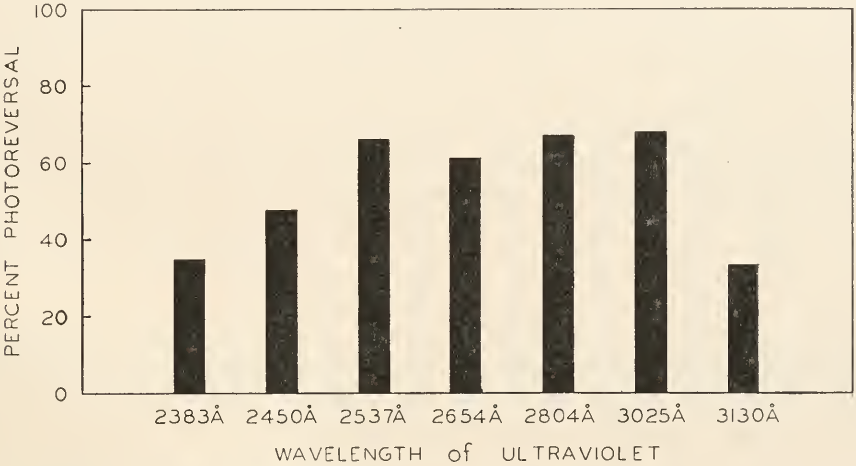

FIGURE 6. Degree of photoreactivation by blue light (56,900 ergs/mm².) after a dosage of 1000 ergs/mm². at each of the UV wave-lengths specified.

DISCUSSION

UV affects division of Colpidium in two ways: a lag appears before division occurs, and, subsequent to larger doses, the rate of division is reduced. Treatment with visible light (or long UV) to a considerable extent reverses both of these effects. UV treatment also caused a lag before division starts and a decrease in division rate in *Paramecium caudatum* (Giese, 1939) and in *Blepharisma undulans* (Giese and Reed, unpublished and Hirshfield and Giese, unpublished). In some ciliates, however, UV treatment also induced a cessation of division which occurs after one, sometimes after two, and, rarely, after three divisions have occurred subsequent to UV treatment. This was seen in *P. aurelia* and in *P. multimicronucleatum* (Giese and Reed, 1940; Kimball and Gaither, 1951). A similar effect has recently been found in *Tetrahymena geleii* (E. Christensen, unpublished), but it was never observed in Colpidium. The reason for this difference in effects of UV on different species of protozoa is not clear.

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[4] Colpidia treated with radiations of λ3025 Å were illuminated with only 42,000 ergs/mm². of blue light owing to an error in calibration, discovered on checking the calculation. This may introduce an error of several per cent in the value for this wave-length in Figure 6.

The different degrees of photoreactivation by blue light after irradiation of colpidia with various wave-lengths of the UV indicate that various UV wave-lengths may produce qualitatively different types of effects. This in turn may possibly be interpreted as being due to alteration of different chemical constituents of the cell by different wave-lengths. Thus certain wave-lengths may affect nucleoproteins more than other protoplasmic constituents, whereas other wave-lengths may selectively affect unconjugated proteins. This is most strikingly brought out (Fig. 6) by the relatively lesser degree of photoreactivation of UV injury at λ2383 Å, which produces rather superficial effects upon the colpidia, immobilizing them quite quickly. Immobilization of ciliary action in Paramecium shows an action spectrum with one maximum at λ2800 Å which corresponds to absorption by unconjugated proteins, whereas retardation of division of the cell shows an action spectrum with one maximum at λ2654 Å which corresponds to absorption by nucleic acid (Giese, 1945a). The results therefore suggest that visible light may be primarily concerned with reversal of UV damage to nucleoproteins or nucleic acid.

For photoreversal of UV injury in Colpidium, the most effective wave-lengths are similar to those found for *Streptomyces griseus* in which a maximum is found at λ4360 Å (Kelner, 1951). For *Escherichia coli,* and a phage which lives upon it, maximum photoreactivation is observed at about λ3650 to 3750 Å (Dulbecco, 1950; Kelner, 1951). Unfortunately the work on Colpidium is not precise enough to enable one to determine a detailed photoreactivation action spectrum. The main uncontrollable variable is the change in physiological state of the colpidia during the growth cycle. If one could obtain animals in exactly the same physiological state, this could be avoided. However, it is difficult to get experimental animals in exactly the same phase in the growth cycle since after a few divisions, the progeny of a single Colpidium begin to divide at a rhythm quite different from one another. The physiological state may have a profound effect on sensitivity to UV. Thus retardation of the third division of a vigorous group of colpidia (*e.g.,* those in which the three divisions occur within 20 to 22 hours in the control) by exposure to a dosage of 1000 ergs/mm^2. at λ2654 Å may be only 20 hours, whereas less vigorous colpidia (*e.g.,* those in which the three divisions occur in the control within 24 to 26 hours) given a similar dosage of UV may show a retardation of the third division of about two to three times the first group. Fortunately for experiments on photoreactivation, the dose reduction is similar; that is, the relative degree of recovery induced by visible light is similar regardless of the UV sensitivity of the colpidia. In recent experiments in which the colpidia were systematically starved before exposure to UV, great increases in sensitivity to UV have been observed with starvation

(Giese, Jacobson and Shepard, unpublished). The nature of the cellular change which causes such a striking difference in sensitivity has not been determined.

Since very large dosages of visible light are necessary for photoreversal of UV injury in Colpidium, it would appear that the substance or bond absorbing the visible light and bringing about the photoreversal is present in very small quantities or that the reaction is very inefficient because of the small amount of energy available in quanta of visible light. Since photodesensitization is so slight in Colpidium, it would appear likely that the compound concerned with photoreactivation appears in the form in which it can fruitfully absorb visible light only after absorption of UV. Shugar's studies (1951) suggest that one cellular compound absorbing light in the region of the spectrum effective in photoreactivation may be the enzyme D-glyceraldehyde-3-phosphate dehydrogenase, present with its reduced coenzyme. Absorption of the light by the adsorbed coenzyme results in reduction of the enzyme; light is ineffective if chemical reduction of the enzyme by cysteine is first performed.

Good photoreversal of UV injury in Colpidium by treatment with blue light even four hours after irradiation with UV light indicates that the injurious substance formed by UV light has a long life and that it does not exert its effect until some time after its production. At first sight this seems to be quite contrary to what has been found in bacteria (Kelner, 1949; Novick and Szilard, 1949). However, a normal Colpidium divides only about once every seven to eight hours at 26° C., whereas bacteria may divide every 30 to 60 minutes. When the inter-divisional time is large, the time during which photoreaction of UV injury can be achieved may also be long.

SUMMARY

1. *Colpidium colpoda*, grown on a single strain of bacteria (*Pseudomonas ovalis*) in 0.05% lettuce medium buffered at pH 7, was irradiated with various wave-lengths of monochromatic ultraviolet (UV) light. UV treatment produces a characteristic lag before beginning of fission and, following larger dosage, a characteristic decrease in division rate. The greatest retardation of division was observed following exposure to λ2537, 2654 and 2804 Å.

2. The retarding effects of UV light at each wave-length were progressively greater with increasing dosages until division of treated colpidia was prevented. A dosage of 3000 ergs/mm^2. at λ2654 Å prevented division and resulted in death of 70 per cent of the colpidia.

3. Illumination with visible light of λ4350 Å effectively reversed UV injury resulting from a dosage of 1000 ergs/mm^2. at each of a number of wave-lengths of UV. For photoreactivation a dosage of 56,900 ergs/mm^2. of blue light (4350 Å) was used.

4. The degree of photoreversal by blue light of injury produced by wave-lengths 2537, 2654, 2804 and 3025 Å was larger than for wave-lengths 2383, 2450 and 3130 Å.

5. Pretreatment with blue light before exposure to UV light did not protect the colpidia; in other words a lag in, or retardation of, division comparable to that found for animals given the UV treatment alone was observed. However pretreatment with bright daylight protected colpidia to some extent from subsequent UV exposure.

6. Wave-lengths 3350, 3660 (long UV), 4050 (violet), and 4350 Å (blue) were effective in photoreversal of UV-injury in colpidia, but the yellow and green portions of the mercury arc spectrum were ineffective.

7. A large degree of photoreversal of UV-injury in colpidia could be induced even four hours after UV treatment, indicating persistence of the UV effect.

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THE SIGNIFICANCE OF THE DARK PERIOD IN THE PHOTOPERIODIC RESPONSE OF MALE JUNCOS AND WHITE-THROATED SPARROWS[1]

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With a very few notable exceptions, all published studies on animal photoperiodism assign the critical role, either explicitly or implicitly, to the length of the daily period of light, acknowledging only the contributory roles of such attributes of light as wave length and intensity, and testing only such possibly modifying factors as temperature, food and activity. This conventional point of view quite disregards the existence of an accompanying, inverse variable, namely, the length of the daily period of darkness.

It is now well established that the length of the period of darkness is critically important in the photoperiodic responses of plants. So-called "long-day" plants, which flower on a relatively long daily period of light, do not *require* the long light period for flowering, since they will flower on a "short-day" if the long period of darkness defining the short day is interrupted by even a brief period of light (*e.g.*, 50 foot-candle minutes; Borthwick *et al.*, 1948).

Of the few studies on animals which incorporate data pertinent to this point, the earliest is that of Shull (1929) on aphids. He found that on a 12-hour day (12-hour night) almost all of the progeny produced by his aphids were winged, whereas on a 14-hour day (10-hour night) practically all the offspring were wingless. But he obtained the same sharply differentiated responses by exposing the aphids to 6-hour periods of light alternating either with 12-hour or 10-hour periods of darkness. Since the length of the light period was identical in the latter two cases, the difference in response must be ascribed to the difference in the length of the dark periods. In Dickson's (1949) extensive study of the photoperiodic control of diapause in the oriental fruit moth, he concluded that although the length of the light and the dark periods are both important factors, in nature diapause is caused mainly by the number of hours of darkness passing a critical minimum point. One of us (Jenner, 1951) has shown that on a short day certain snails fail to lay eggs, or lay very sparingly, but if on a shorter day the dark period is interrupted with light (total light per 24 hours equal to or less than the short-day controls), eggs are laid as abundantly as when the snails are on a long day. He has since obtained comparable results with the fresh-water shrimp, *Palaemonetes*. Hammond (1951) found that on a schedule of 7 light-hours alternating regularly with 5 dark-hours female ferrets come into oestrus ahead of ferrets exposed to normally increasing winter day-lengths; this schedule might possibly be interpreted as a combination of short days (7 hours) with short nights (5 hours), but obviously there may be other very different interpretations. Hart (1951), also using female ferrets, found that by adding just one hour of light at midnight to the normally increasing day-lengths of winter the oestrus cycle was greatly accelerated. However, due to lack of an adequate control group, it cannot be determined whether his results are to be ascribed to the extra hour of light or to the interruption of the dark period.

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[1] An abstract of this paper appeared in the Anatomical Record, volume 113 (1952).

To the best of our knowledge, there are no other published data bearing on this problem in animals, either invertebrate or vertebrate, and it is apparent that, for vertebrates at least, the evidence is not only meager but at most inconclusive.[2] The experiments to be described below were designed expressly, then, to answer the question: Is it the length of the daily period of light alone which is critical, as has been generally supposed, or does the length of the daily period of darkness play a definitive role in the photo periodic mechanism of vertebrates?

Materials and Methods

Slate-colored juncos (*Junco hyemalis*) and white-throated sparrows (*Zonotrichia albicollis*) were trapped during the winter of 1951–52 in the Arboretum on the university campus and on the grounds of the zoology building, and were distributed in six cages, six to eight birds in each cage. A 40-watt, 48-inch daylight fluorescent tube was suspended about one foot above each cage; these lights were turned on and off by automatic 24-hour clock timers. The cages were arranged in pairs, back-to-back; one cage of each pair contained juncos, the other held sparrows.

Each pair of cages was in a separate room, two of which were light-tight; in the third room the lights went on before dawn and remained on until after dark, for a total of 16 hours. The birds in this latter room are referred to as Group A (long day—short night) or long-day controls. In one of the light-tight rooms, the lights were on continuously for 10 hours in each 24; during the other 14 hours the birds were in complete and continuous darkness. These birds are referred to as Group B (short day—long night) or short-day controls. In the second light-tight room, the lights were operated by two timers in such a way that the dark period following 8¼ hours of continuous light was interrupted by a second light period of 1¾ hours. Thus, although the total hours of light per 24 were the same as in Group B, namely 10 hours, the periods of continuous darkness were only 7 hours rather than 14 hours as in B. Birds from this room are referred to as Group C (short day—interrupted night). These three light schedules are shown diagrammatically in Figure 1. The lights delivered 25–35 f.c. on the floor and perches. Group A birds were exposed occasionally to higher and more variable intensities.

The cages were constructed entirely of ¼-inch hardware cloth, and measured 2 ft. × 2 ft. × 3 ft. Each was provided with two perches made of ¼-inch dowelling, 2 feet long and placed about 5 inches above the floor of the cage, which rested on small wooden blocks.

Fine-cracked corn was present at all times in the food trays, which also contained a commercial "bird gravel." Pablum was supplied daily in a separate dish; both species seemed to prefer this to the grain. During most of the experimental period peanut hearts were mixed with the grain, as well as small amounts of a commercial "bird seed." A continuous supply of water was assured by the use of quart jars inverted on baby-chick watering pans. The birds appeared to remain healthy under these conditions; all that were sacrificed in the course of the experiment were at least somewhat fat. Apart from a few juncos that died within 12 hours of capture, only four birds died in the cages out of a total of 38 caged birds.

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[2] While this paper was in press, Kirkpatrick and Leopold (Science, volume 116, pp. 280–281) reported the induction of full sexual activity in quail held on short day—interrupted night light schedules essentially similar to those employed by us, and concluded, as we do, that "the duration of the dark period is a major controlling factor of photoperiodic responses."

Maximum and minimum temperatures were recorded daily in each room, from thermometers placed next to the floor of each pair of cages. The temperature record (° F.) is as follows:

	Average maximum	Average minimum
A	74.5 (67–81)	66.5 (60–72)
B	75.2 (72–79)	71.6 (69–76)
C	73.5 (71–77)	70.0 (67–74)

The slight differences, especially as between the control room B and experimental room C, are not regarded as significant (cf. Burger, 1948).

Groups A and B were in our personal laboratories, group C in a small, otherwise unoccupied room. To avoid disturbance to the birds the A and B cages were partly shrouded with cloth draped from the frame supporting the lights, and this effectively shielded the birds from sight of movements in the room. They did not appear to notice the sound of conversation or other noises; the birds of group A, especially, frequently sang throughout the day. Since the results place group B in sharp contrast with both A and C, the isolated and relatively undisturbed position of the C cages is not regarded as relevant.

Juncos were trapped on January 4, 5 and 6, 1952, banded and placed under treatment on the dates of capture. All the juncos were killed on March 1, after 55–57 days of treatment. The initial group of white-throated sparrows was trapped during the period December 17–20, 1951, and the experiment begun on December 19. Additional sparrows were trapped and added to the cages between December 30 and January 3. These latter birds were banded to distinguish them from the original, unbanded lot, but the loss of some bands in the C cage made uncertain the length of treatment for the first C birds killed, on January 17. The sparrow experiment was terminated on February 21. Birds were classed as adults or immatures on the basis of the frontal bones (Miller, 1946) at the time of sacrifice. It is possible that some which were "immatures," *i.e.*, birds of the year, at the start of the experiment had become "adults" on this criterion by the end of the experimental period.

The birds were killed with chloroform, in groups of three to six. Both testes of each male were removed with fine forceps, placed together in a tared weighing bottle, weighed on a balance to the nearest 0.1 mg. and then immediately flooded with Bouin's fluid. Long and short diameters of each testis were subsequently measured for determination of volumes, and one (usually the larger) of each pair was sectioned at 10 μ; the sections were stained with Ehrlich's hematoxylin, some counterstained with eosin. The volumetric data in Tables I and III refer to the sectioned testis, weights to the pairs of fresh testes. Maximum tubule diameters were measured from the sections with an ocular micrometer, and the progress of spermatogenesis recorded according to the stages of Blanchard (Blanchard, 1941;

TABLE I

Some data on results of artificially imposed lengths of day and night on male slate-colored juncos (Junco hyemalis)

Date collected (1952)	Duration of treatment (days)	Weight both testes (mg.)	Volume sectioned testis (mm.^{3})	Max. tubule diam. (μ)	Stage of spermatogenesis
Field Controls					
Jan. 6	—	1.6	0.64	55	I
Jan. 8 (immature)	—	0.6	0.31	52	I
Jan. 11 (immature)	—	0.4	0.19	42	I
Feb. 28	—	4.7	2.05	100	IV
Feb. 28	—	1.4	0.47	75	II
Mar. 5	—	1.2	0.68	65	II
Mar. 6	—	1.9	1.06	82	III
Mar. 6	—	2.3	0.82	78	III
Mar. 6	—	1.8	0.74	75	III
Mar. 6	—	1.4	0.74	88	III
Mar. 6	—	0.6	0.58	82	III
Mar. 6	—	0.8	0.43	75	III
Group A. "Long day—short night"					
Jan. 4	57	348.8	205.0	575	VII
Jan. 4	57	188.5	93.0	525	VII
Jan. 5	56	245.2	139.0	600	VII
Jan. 5	56	160.1	94.0	500	VII
Jan. 6	55	184.8	107.0	450	VII
Group B. "Short day—long night"					
Jan. 4	57	0.9	0.52	78	III
Jan. 4	57	0.3	0.23	58	II
Jan. 5	56	1.6	0.76	91	III
Jan. 5	56	0.7	0.38	72	II
Jan. 6	55	0.8	0.42	65	II
Group C. "Short day—interrupted night"					
Jan. 4	57	128.5	70.5	400	VII
Jan. 4	57	76.2	37.5	350	VI
Jan. 4	57	50.8	25.5	300	VI
Jan. 5	56	77.7	42.5	375	VI
Jan. 5	56	4.2	1.84	125	IV
Jan. 6	55	18.2	8.10	200	V

Blanchard and Erickson, 1949). These stages may be characterized, briefly, as follows:

I. Inactive; interstitial lipoid Leydig cells absent, or, if present, small, not recognizable in ordinary preparations.
II. First appearance of recognizable Leydig cells.

III. Marked increase in number of spermatogonia; Leydig cells numerous.
IV. A few primary spermatocytes in synapsis in each tubule cross-section.
V. Predominance of primary spermatocytes in synapsis.
VI. (early). Predominance of spermatids.
VI. (late). Spermatids in transformation, much cellular debris in lumen of tubule, many sperm bundles but fewer sperm per bundle than in fully mature condition.
VII. Breeding condition; tubules packed with sperm bundles; mature sperm free in lumen.

RESULTS

Slate-colored juncos (*Junco hyemalis*). (Fig. 2; Tables I and II). The field controls demonstrate that at the beginning of the experiment the testes were minute and spermatogenically inactive.

Group A. After 8 weeks on a "long day—short night" schedule, spermatogenesis was complete in each of the five males in this group. The tubules were swollen, each with a large lumen, and packed with bundles of mature sperm.

Group B. Recrudescence of the testis was initiated in each of the five males on a "short day—long night" schedule after 8 weeks. However, none had developed beyond the stage of marked increase of spermatogonia (stage III) and only two had even reached that stage. The testes were still minute, but the tubules had enlarged slightly.

Group C. After 8 weeks on the experimental "short day—interrupted night" schedule, testes of four of the six males contained fully developed sperm in numerous bundles. One of these four corresponded to the long-day group A testes, with spermatogenesis complete; in the other three many spermatids were still in the process

TABLE II

Stages of spermatogenesis attained by slate-colored juncos, artificially lighted from Jan. 4–6 to Mar. 1. Each "x" indicates one bird

Stages		Field Jan. 6–11	A Long day	B Short day	C Interrupted night	Field Feb. 28–Mar. 6
VII			xxxxx		x	
VI	late				xxx	
	early					
V					x	
IV					x	x
III				xx		xxxxxx
II				xxx		xx
I		xxx				

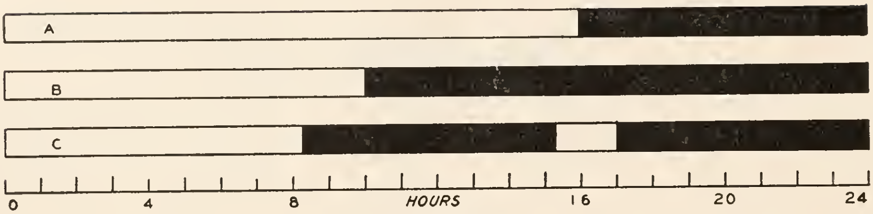

FIGURE 1. Schematic diagram of light schedules per 24 hours. A = long-day control; B = short-day control; C = "interrupted night." Total number of light hours in C the same as in B, but through interruption of darkness in C, periods of continuous darkness are one hour less than in A.

of metamorphosis, although there were many small bundles of metamorphosed sperm in each tubule section. In the testes of the remaining two experimental birds the first maturation division was under way.

Most of the juncos field-trapped and sacrificed at about the termination of the experiment were in spermatogenic states comparable to those attained by our short-day controls, group B. Only one of nine had reached the stage of initiation of spermatocyte division. That one is thus similar to the least developed of our experimental birds of group C. At this time, day lengths were approaching 12 hours (sunrise-sunset), and these outside birds had been exposed, for almost four weeks, to day lengths exceeding the 10 hours allowed our experimental and short-day control birds.

White-throated sparrows (*Zonotrichia albicollis*). (Tables III and IV.) The results, at first glance, may not appear to be as convincing as in the case of the juncos.

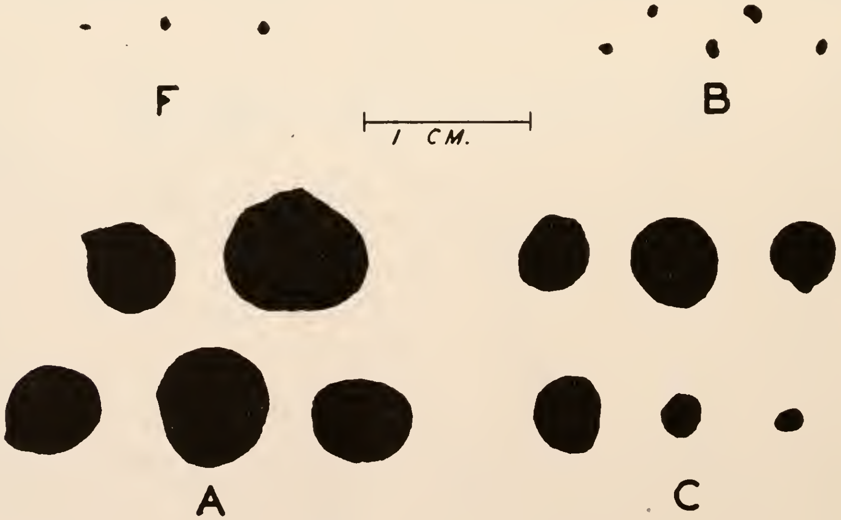

FIGURE 2. Relative sizes of junco testes in early January (F = field controls) at start of experiment, and after 55–57 days on the experimental light schedules of Figure 1; each testis is from a different bird. From a negative photographic print. A = long-day control; B = short-day control; C = "interrupted night."

Most of the birds were sacrificed at the end of four weeks, a date which proved to be perhaps too early, and left very few birds to be treated for seven weeks. Then, due to the loss of some bands, the group C birds sacrificed at the end of four weeks included a few undetermined individuals that had been in the cages only 15–18 days. Nevertheless, we believe, especially in the light of the results with juncos, that these

TABLE III

Some data on results of artificially imposed lengths of day and night on male white-throated sparrows (Zonotrichia albicollis)

Date collected (1951–52)	Duration of treatment (days)	Weight both testes (mg.)	Volume sectioned testis (mm.^{3})	Max. tubule diam. (μ)	Stage of spermatogenesis
Field Controls					
Dec. 29	—	1.9	1.01	58	I
Dec. 29	—	2.1	0.98	55	I
Dec. 29	—	2.2	0.89	55	I
Dec. 29 (immature)	—	0.5	0.42	46	I
Feb. 28	—	4.0	3.04	68	II
Feb. 29	—	5.5	2.35	65	II
Feb. 29	—	0.6	0.55	49	I
Group A. "Long day—short night"					
Dec. 17–20	29–30	161.0	87.0	420	VII
Dec. 17–20	29–30	100.9	47.9	330	VI
Dec. 17–20	29–30	81.0	31.4	390	VI
Dec. 30	52	274.1	200.0	650	VII
Jan. 1	51	151.5	67.5	520	VII
Jan. 3	49	258.7	143.5	600	VII
Group B. "Short day—long night"					
Dec. 17–20	29–30	2.7	1.20	68	II
Dec. 17–20	29–30	2.7	1.06	65	I
Dec. 17–20	29–30	1.9	1.06	71	II
Dec. 17–20	29–30	1.9	0.95	62	I
Dec. 17–20 (immature)	29–30	—	1.08	65	I
Jan. 1	51	0.5	0.34	58	II
Jan. 3	11*	1.9	0.95	62	II
Group C. "Short day—interrupted night"					
Dec. 17–20	29–30	5.6	3.32	81	III
Dec. 17–20	29–30	3.8	1.20	62	II
Dec. 17–20 (immature)	29–30?	2.3	0.95	81	III
Dec. 17–20 (immature)	29–30	1.9	0.78	71	II
Dec. 17–20 (immature)	29–30	1.2	0.84	68	II
Jan. 3	49	52.1	31.2	290	VI
Jan. 3	49	9.4	10.3	160	IV

* = died in cage; ? = some of these collected Dec. 20–Jan. 3, treated only 15–18 days (bands lost).

data for white-throated sparrows do show that the interruption of the long dark period resulted in an acceleration of the spermatogenic process. At the beginning of the experiment (field controls), the testes of all birds were minute and inactive.

Group A. On a "long day—short night" schedule, the testes of three birds had greatly increased in size after four weeks. In one of these the tubules were packed with sperm, as in the breeding condition; in the other two the tubules were greatly enlarged, with many spermatids and a few sperm. After 7 weeks, the testes of three additional males had reached full breeding condition.

Group B. On the "short day—long night" regime, after four weeks, the testes of the three most advanced males showed just the beginning of recrudescence; in three others, the testes still were inactive. Even after 7 weeks, the single male left in this group had not advanced beyond the earliest stage of recrudescence (first appearance of interstitial Leydig cells).

Group C. Of the experimental males on a "short day—interrupted night," after four weeks the testes of one were definitely enlarged, and spermatogenesis had reached the stage of marked increase of spermatogonia. In another male the testes were still small, but the tubules showed a definite although slight increase in diameter and spermatogenesis had likewise reached the multiplication stage (III). The other three males had begun recrudescence. No testis was still in the inactive state, despite the fact that some of these birds (individuals unknown) had been treated for only 15–18 days. Two males were treated for 7 weeks; in both of these the testes had increased very considerably in size. One had reached the penultimate stage of spermatogenesis, with swollen tubules packed with spermatids; in the other some spermatocytes were beginning to divide. This latter condition is the one

TABLE IV

Stages of spermatogenesis attained by white-throated sparrows under artificial lighting

Stages		Field Dec. 29	A Long day	B Short day	C Interrupted night	Field Feb. 28–29
VII			a bbb			
VI	late					
	early		aa		b	
V						
IV					b	
III					aa	
II				aaa b	aaa	xx
I		xxxx		aaa		x

"a", "b" and "x" each denote one individual; "a" = experimental period of 29–30 days (Dec. 19–20 to Jan. 17), and "b" = experimental period of 49–52 days (Dec. 30–Jan. 3 to Feb. 21).

which, in a related species, either immediately precedes the beginning of spring migration, or, in non-migratory populations, accompanies pairing and onset of territorial behavior (*Z. leucophrys*, Blanchard, 1941; Blanchard and Erickson, 1949).

It is unfortunate that only one male was left in the control group B after seven weeks; but it is noteworthy that several days after the termination of the experiment three males collected in the field were far behind the less mature of the last two experimental birds. As explained above under juncos, the outside birds had been exposed to considerably more light per 24 hours than our groups B and C.

Discussion

Among our birds on the short day—interrupted night schedule, full testicular development was obtained not by exposing the birds to extra light but by breaking the dark period into two "short nights." It is therefore concluded that the length of the uninterrupted period of darkness is a controlling factor. Darkness evidently is not neutral or merely indifferent in the activation or inactivation of the photoperiodic response. Some sort of reaction must be going on in the dark which, probably, bears an inverse relationship to the reaction going on in light. If the dark period is long enough gonadotropic activity does not occur. If the dark period is short (or absent) gonadotropic activity does occur, but its occurrence is independent of the *length* of the light period, providing only that the duration of the light period is greater than some relatively low minimum. The significance of the length of the photoperiod is related primarily to the way in which it defines the length of the dark period. The species used in our experiments, and undoubtedly some other passerine birds as well, certainly do not require at least 11 to 12 hours daily light to complete spermatogenesis, as the conventional view would have it. What they do require, apparently, is that the daily dark period be less than 12 or 13 hours.

In our experiments the short day—interrupted night schedule obviously was not as effective in stimulating spermatogenic advance as was the long day—short night schedule, even though the dark periods (7 hrs.) were shorter than the dark period (8 hrs.) of the long day. Further investigation will be necessary to explain this difference. In our opinion the probable explanation is that the reaction which occurs in the light reaches a maximum at a quantity of light (intensity × time) greater than that employed for the dark-period interruption in our experiments. The fact that the testes of four of the six juncos on the interrupted-night schedule developed mature sperm (late stage VI or VII) in 8 weeks would seem to indicate that the interruption given (1¾ hours at about 30 f.c.) approached the light energy required for complete effectiveness. Therefore, if we consider that a light quantity slightly greater than 1¾ hours at 30 f.c. represents the order of magnitude of the light energy required to complete the light phase of the photoperiodic response, then it is obvious that in our experiments the light reaction reached the same maximum on the short day of 10 hours (group B) as on the long day of 16 hours (group A). The difference in response between these two day lengths must then be due to the difference in the length of the dark periods. We interpret this to indicate that whereas the light-phase of the reaction reaches a maximum rapidly, the dark-phase reaction takes place very slowly, and is therefore the time-measuring phase of photoperiodism.

There is an extensive literature on photoperiodism in birds and mammals. Conventionally it is thought that in these responses ". . . the light is received by the eye which sets off an unknown sequence of events terminating in stimulation of the pituitary" (Burger, 1949; p. 218). The pituitary in turn produces the gonadotropins which stimulate the gonads. The experiments reported here direct attention to what appears to be a characteristic feature of all photoperiodic responses, namely, that the duration of the dark period is critically important. The conventional view of animal photoperiodism must therefore be amended to include this concept.

The results of the present experiments, like those of Jenner (1951), demonstrate a similarity between plants and animals in their photoperiodic responses. The recognition of such a pattern of similarity emphasizes the desirability of understanding its basis.

SUMMARY

1. Experiments were conducted with slate-colored juncos and white-throated sparrows to test the thesis that the dark period plays a definitive role in the photoperiodic response of these birds.

2. The results of the experiment using male juncos were as follows:

(a) Testes of birds on a short day (10 hours of light, 14 hours of darkness) underwent little or no development during the 8 weeks of the experiment.

(b) Exposure of other juncos to a long day (16 hours of light, 8 hours of darkness) during this same period resulted in their testes attaining full breeding condition.

(c) Experimental birds were placed on a light schedule which consisted of 8¼ hours of light followed by a dark period, the middle of which was interrupted by a second light period of 1¾ hours; thus they received a total (as in (a)) of 10 hours of light per 24-hour period, but dark periods were only 7 hours long. By the end of 8 weeks considerable testicular development had occurred among all males placed on this "interrupted-night" schedule. The testes of four birds had developed mature sperm (late stage VI or VII); the other two were somewhat less developed.

3. The response of white-throated sparrows placed under these same conditions was similar to that of the juncos.

4. The results show clearly that in the photoperiodic response of these birds there is a critically important dark-dependent phase. This dark-period dependence appears to be characteristic of all photoperiodic responses, in both plants and animals.

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ON THE RELATION BETWEEN WATER TRANSPORT AND FOOD REQUIREMENTS IN SOME MARINE FILTER FEEDING INVERTEBRATES

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Filter feeding invertebrates in the sea, *e.g.,* sponges, ascidians, most lamellibranchs, several gastropods and crustaceans, etc., obtain their food from the finely dispersed organic matter which is present in the water. A minor fraction of this organic matter is particulate and can be removed from the water by means of suitable filters, whereas a major fraction is "dissolved." It is not finally settled whether filter feeders obtain their food from the particulate fraction only, or whether they are also able to utilize the so-called dissolved organic matter in the sea. The view that filtering animals utilize the dissolved substances is mainly supported by calculations showing that particulate organic matter is not present in sufficient amounts to cover the energy requirements of the filter feeders. The calculations are based on measurements or estimates of rate of water transport, oxygen consumption and growth of the animals in question. The calculations are, however, somewhat uncertain because in no instance have water transport and metabolic rate been determined in the same specimen. It was therefore decided to measure filtration rate and oxygen consumption in the same individuals of some filter feeders and to repeat the calculations mentioned above.

TECHNIQUE

The species used were the oyster, *Ostrea virginica,* and the ascidians *Ciona intestinalis* and *Molgula manhattensis.* Experiments were performed on one oyster at a time or on 5 Ciona or about 15 Molgula. The oysters were kindly supplied by Dr. Paul S. Galtsoff, Shellfish Laboratory, U. S. Fish and Wildlife Service, Woods Hole. They had been living in the tanks of the laboratory for about a month. Ciona and Molgula were used in the experiments after they had been adapted to the laboratory conditions for a day. Filtration rates were determined as previously described (Jørgensen, 1949a) by measuring the rates at which graphite particles ("Aquadag A") were removed by the animals. Graphite concentrations were measured by means of a Klett-Summerson photoelectric colorimeter. Control experiments showed that the particles used were retained almost completely in the filtering organs (Jørgensen and Goldberg, unpublished data). Oxygen was determined by the Winkler method.

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[1] Lalor Fellow at the Marine Biological Laboratory during the summer of 1951.

RESULTS

The results of the experiments are shown in Figures 1–4. The points in the graphs represent single determinations of filtration rate or oxygen consumption in liters of water or milliliters of oxygen per hour. It is seen that the rate of water transport was of the same order throughout the period of observation which lasted three to four days. In Ostrea and Molgula, the water transport was approximately

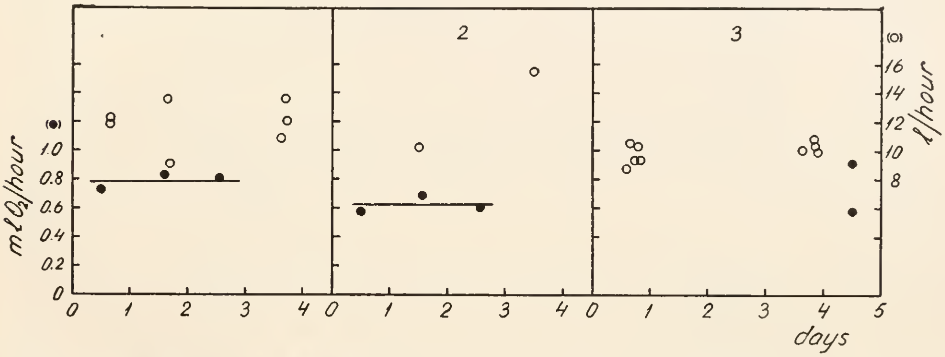


FIGURE 1. *Ostrea virginica.* ● Oxygen uptake in ml. per hour. ○ Filtration rate in liters per hour. Experiments on three individuals.

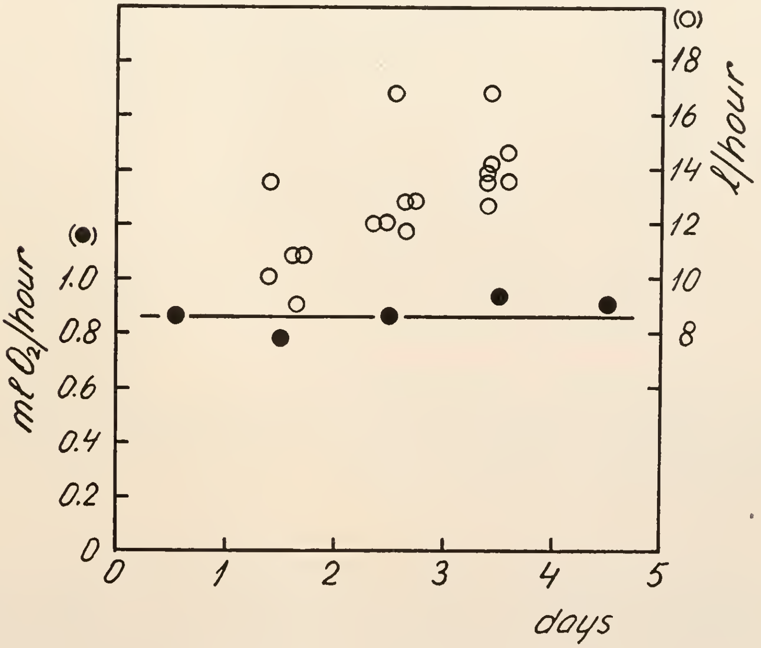
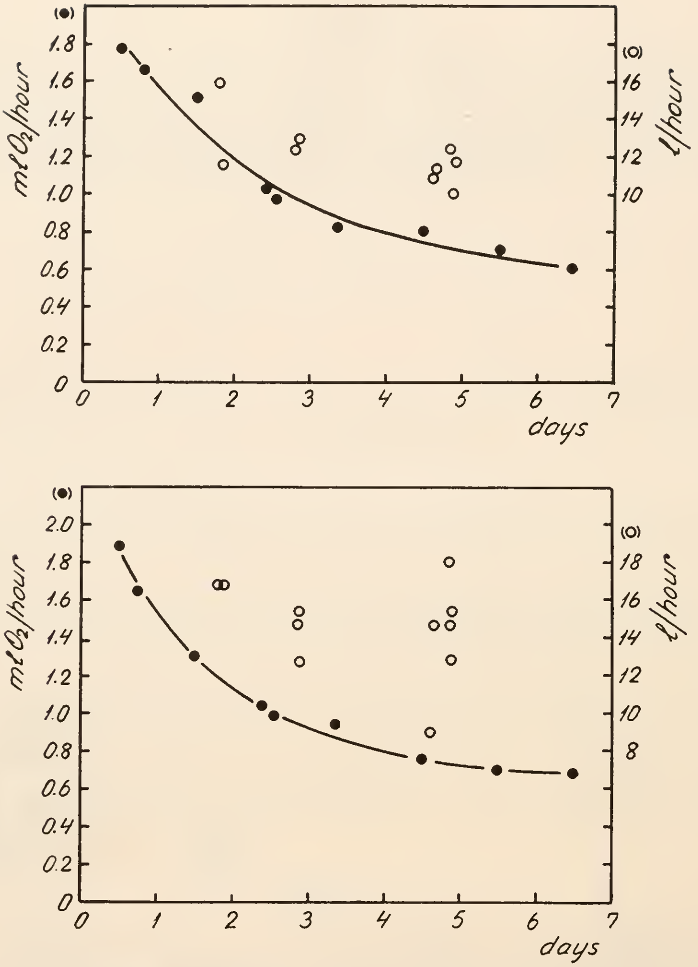


FIGURE 2. *Ciona intestinalis;* 5 specimens. ● Oxygen uptake in ml. per hour. ○ Filtration rate in liters per hour.



FIGURES 3 AND 4. *Molgula manhattensis.* About 15 specimens. ● Oxygen uptake in ml. per hour. ○ Filtration rate in liters per hour.

constant from day to day. In Ciona, filtration rates were lower the first day than the two following days, probably because the animals became better adapted to the experimental conditions with time. The oxygen uptake was constant in the oyster and Ciona, but decreased with time in Molgula.

DISCUSSION

From Figures 1 and 2 it is seen that the oyster and Ciona pumped about 10 to 20 liters of water through their filtering organs for each ml. of oxygen uptake. In Molgula the ratio between water transport and metabolic rate is not well defined because oxygen was consumed at decreasing rates throughout the experiments. There is reason to believe that at least two factors are responsible for the decrease in oxygen uptake. On arrival in the laboratory hydroids, bryozoans, algae, etc. growing on the mantle of the animals were removed. This procedure, which also involved separation of adhering animals, may have caused an increase in metabolic rate so that the rates of oxygen uptake have perhaps been abnormally high during the first days of the experiments. Mytilus when detached from their support by tearing of the byssus threads were also found to respond by strongly increasing their oxygen consumption. Thus, the steady decrease in metabolic rate of Molgula may just indicate a slow return towards values of oxygen uptake characteristic of undisturbed animals. However, poor food conditions may also be partly responsible for the continued decrease in oxygen consumption as pointed out below. Probably, therefore, normal metabolic rates are to be found somewhere in between the rates which were measured at the beginning and at the end of the experiments. If the measurements made on the third day of the experiments are used as "normal" values we find also in Molgula that about 10 to 20 liters of water are filtered for each ml. of oxygen uptake. Filtration rates of the same order were also measured in *Mytilus edulis*, *Pecten latiauritus* and *Ciona intestinalis* from coastal waters off Southern California (unpublished data).

The literature contains data on feeding rate and oxygen uptake in other filter feeders. Even when these data are not obtained from the same individuals and under identical experimental conditions, they are consistent with the results of the present study.

In the copepods *Calanus finmarchicus* and *Centropages hamatus*, feeding rate and oxygen uptake have been measured by Gauld (1951) and Raymont and Gauld (1951), respectively. From their figures, filtration rates of about 8 to 10 liters of water per ml. of oxygen uptake can be calculated.

Jørgensen (1949b) found that the silicious sponge *Halichondria panicea* filtered 65 ml. of water per hour and per mg. nitrogen (18–19° C.). Pütter (1914) measured the oxygen uptake in another silicious sponge, *Suberites massa,* to be 0.34 ml. per hour and per gram dry organic matter (22° C.). Assuming a content of 8 per cent nitrogen, this means 4.2 ml. O_2/hour/mg. nitrogen. Thus, if these values for water filtration and oxygen uptake are generally valid in silicious sponges, 15 liters of water are filtered for each ml. of oxygen uptake. The calcareous sponges *Grantia compressa* and *Sycon coronatum* showed pumping rates of about 140–200 ml./hour/mg. nitrogen (Jørgensen, 1949b). Probably, therefore, their filtration rate relative to metabolism is of the same order as that of the silicious sponges, perhaps even higher.

In phylogenetically unrelated filter feeders which use different filtering mechanisms, the rates of filtering and the metabolic rates have been adjusted to about the same ratio. Presumably the feeding rate, not the filtration rate as such, is primarily adjusted relative to the metabolic rate (and relative to the rate of growth). A constant ratio between rates of filtration and of oxygen uptake in different filter feeders therefore suggests that the concentration of available food in the sea where adaptation took place was about the same for all the filter feeders mentioned above. This conclusion of course rests on the assumption that the rate of filtration of the animals is the same in their natural environment as under the conditions of experiment. However, there is some reason to believe that this assumption holds. Food conditions must be better in the sea than in the running tap water of the laboratory where much food is removed by other organisms growing in storage tanks and water pipes. The decrease in oxygen uptake observed in the course of an experiment in Molgula (Fig. 3 and 4) and in Mytilus and Pecten (unpublished) is probably partly due to starvation. However, filtration rates were not influenced by the reduction in food concentration. Filtration rates of lamellibranchs and ascidians are also independent of increased concentrations of food and other particles as long as the concentrations are not too high (Loosanoff and Engle, 1947, and others). Thus, if filtration rate can adjust to different food levels, this process must be slow. Hence, when measured with a suitable technique [2] the pumping rate and the oxygen uptake in undamaged animals which have been adapted to the laboratory conditions are therefore probably representative of the corresponding rates prevailing in the sea.

"Available food." The organic matter in the sea is present as organisms such as phytoplankton, bacteria, Protozoa, etc., but also as dead material such as decaying plants and animals. The organic matter varies greatly in particle size; phytoplankton cells, for example, range from about one to several hundred micra. The decaying material (detritus), of course, has no well-defined particle size and there is no sharp distinction between particulate and dissolved organic matter. Such a distinction, however often made, must therefore be a rather arbitrary one. "Particulate" material usually means material retained by paper or membrane filters with pore diameters of about one micron, whereas "dissolved" and "colloidal" matter passes through such filters. In this paper particulate organic matter is assumed to have a particle size of about one micron or more. The composition and the physical properties, such as particle size, of "dissolved" or "colloidal" organic matter are practically unknown. The latter is an important fraction, generally amounting to several times the particulate fraction. Values of dissolved organic nitrogen from various parts and depths of the sea range from 0.1 to 0.26 mg. per liter (Krogh and Keys, 1934, and von Brand and Rakestraw, 1941). Dissolved organic carbon is present in an amount of about 2.36 mg./liter. Assuming a mixture of carbohydrate and protein this would mean that about 1.5 mg. protein and 3.9 mg. carbohydrate are dissolved per liter of sea water (Krogh, 1934). The amount of particulate organic matter, living or dead, varies more than does that of dissolved matter. Some figures representative of coastal waters are given in Table I. Values from open sea and from great depths are smaller. However, the figures from coastal waters are of special interest to us because all determinations of filtration rates in filter feeders are gained in such areas.

It has often been discussed whether sufficient particulate organic matter is present in the water to cover the food requirements of filter feeders or whether dissolved or colloidal matter has to be taken into consideration as an additional resource. This discussion will not be reviewed here in detail. It should only be pointed out that the different approaches to the problem have led to contradictory results. One approach has been to estimate how much water should be filtered free of particulate food in order to meet the energy requirements of the animal in question. Pütter (1909, 1914, 1925) made extensive studies on a variety of marine animals and concluded that the necessary filtration rates were beyond reasonable values. Fox and Coe (1943) made similar calculations for *Mytilus californianus.* They state that the quantity of micro-organisms present in the water does not provide enough nutriment for growth and metabolism of the mussel. They therefore conclude that about 5 mg. of organic matter, found by Krogh to be in solution in sea water, must be accessible to the mussels as food. On the other hand, direct attempts to demonstrate the importance as food of dissolved organic substances, either added or naturally occurring in the water, turned out negative (Krogh, 1931; Bond, 1933; and others).

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[2] By "suitable technique" is meant a technique which does not significantly interfere with the normal filtering activity of the animal. Generally, even slight handling or disturbances of a filter feeder will reduce filtration rate. The values of water transport given in the present paper, as in other papers, are therefore more likely to be too low than too high.

It is of interest to repeat the calculations on the basis of our present knowledge of metabolic, growth and filtration rates, and to relate the results with the quantity of particulate organic matter which can be assumed to be present in the water.

TABLE I

Locality	Phytoplankton mg. organic matter per liter		Author	Remarks
	Range	Average		
English Channel	0.04–0.2 0.01–0.23	0.06	Harvey, 1950 Atkins and Parke, 1951	Annual variation Annual variation
Baltic Sea	0.35		Krey, 1939	March
Long Island Sound	0.17–2.1 0.6 –2.8	0.59	Riley, 1941 Riley, Stommel and Bumpus, 1949	June–October June–September
Block Island Sound		0.38	Riley, personal communication	
Coastal water south of Cape Cod	0.14–0.63		Riley and Gorgy, 1948	July–September
Coastal water off La Jolla	0 –0.22	0.09	Graham, 1943	Annual variation

The calculations are based on a filtration rate of 15 liters for each ml. of oxygen uptake. One ml. of oxygen is required to combust about 0.8 mg. organic matter of mixed food. Fifteen liters of water should therefore contain at least 0.8 mg. organic matter which can be retained and utilized by the animals, or about 0.05 mg./liter. Obviously, organic matter which is used for growth must also be taken into consideration. The efficiency with which absorbed nutrients are utilized for growth varies with the age of the animals. In filter feeders such as lamellibranchs, gastropod veligers or copepods, up to about ⅔ of the energy absorbed can be used for growth (Harvey, 1950, and Jørgensen, 1952). Therefore, the actual food requirements for growth and respiration of the animals investigated probably does not exceed about 0.15 mg. utilizable organic matter per liter of water. The average figures for phytoplankton (Table I) range from 0.06 mg. to 0.59 mg./liter, the lowest figure being found in the English Channel. Here, total organic particulate matter is, however, 1.6–1.8 mg./liter (Armstrong and Atkins, 1951). The values from La Jolla are perhaps too low because, in some instances, very little or no chlorophyll was measured despite rather high counts of chlorophyll-containing algae cells in the water. Thus at least during part of the year, particulate organic matter, phytoplankton and detritus, in the coastal waters is in excess of the amount needed for maintenance and growth of a number of filter feeders. The shortcoming of previous calculations is due mainly to an under-estimation of filtration rates, but also of the amount of particulate food in the sea.

It remains to be demonstrated that the filtering organs of the filter feeders are in fact able to retain what is generally measured as particulate organic matter in the sea, *i.e.,* material with a minimum particle size of about one micron. The efficiency of the filtering organs has been investigated in rather few species only. It was found that *Ciona intestinalis* almost completely retains one-micron particles. The same is probably true of other tunicates as well as sponges. In the lamellibranchs Mytilus, Ostrea and Pecten, the minimum particle size effectively retained varied from about one to a few micra (Jørgensen, 1949a, and Jørgensen and Goldberg, unpublished data). This probably also holds for many copepods (Ussing, 1938). Thus, most of the particulate organic matter in the sea is available to at least a considerable number of filter feeders.

Adjustment of food requirements to rate of food uptake. As mentioned above, changes in the concentration of food particles were not accompanied by changes in filtration rate.[3] The feeding rates therefore decrease with decreasing quantities of food in the water. Perhaps the reduction in metabolic rate to be observed in the course of the experiments with Molgula and other filter feeders can be interpreted as an adaptation to poor feeding conditions. Pütter (1914) observed a similar decrease in oxygen uptake in the sponge Suberites, and it is worth mentioning that v. Brand *et al.* (1948) found striking reductions (up to 80 per cent) in metabolic rates during starvation in a number of pulmonate gastropods.

However, more information is needed before we can obtain a clear picture of the interdependence between rate of filtration, metabolism, growth of different filter feeders and the concentration of food particles in the surrounding water.

SUMMARY

The oyster, *Ostrea virginica,* and the ascidians *Ciona intestinalis* and *Molgula manhattensis* filter about 10 to 20 liters of water for each milliliter of oxygen consumed. It has been demonstrated that this filtration rate is probably great enough to provide the animals with sufficient amounts of particulate food for maintenance and growth.

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APPLICATION OF THE MOST PROBABLE NUMBER METHOD TO DETERMINE HEAT SENSITIVITY OF PROTOZOA

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In order to determine the deleterious effect of specific agents or treatments on populations of micro-organisms, it is desirable to employ an end-point dilution method to determine number of viable survivors. Plating procedures have not been suitable for use with protozoa, except in the case of certain non-motile and pigmented forms. Lack of a suitable method has hampered studies on the sensitivity of protozoa to specific chemical substances or physical treatments. The most probable number method, frequently used by bacteriologists, was adapted for use in a study on heat sensitivity of Tetrahymena, and the results are described below.

MATERIAL AND METHODS

The ciliated protozoan, *T. pyriformis,* strain WB, as designated previously (Loefer, 1952) and used in an earlier investigation (Loefer and Matney, 1952), was employed in this study. Ingredients of the medium were: Bacto-Casitone, 2%; Bacto Yeast Extract, 0.25%; and salts in mg. % as follows: NaCl, 20; $CaSO_4$, 5; $MgSO_4$, 2.5; KNO_3, 20; KH_2PO_4, 10; K_2HPO_4, 10; $FeCl_3$, 0.5; pH, 7.1–7.3. Cultures in their log phase were used for all determinations. Exposure to specific temperatures was effected in 22 × 175 mm. Pyrex tubes in a constant temperature water bath ($\pm 0.01°$ C.). A concentrated suspension of ciliates (0.1 ml.) was introduced into pre-heated media (20.0 ml.) and aliquots were removed aseptically at specific time intervals as indicated and transferred to cool (25° C.) dilution bottles containing 0.1% Bacto-Casitone plus inorganic salt solution, so as to obtain series of ten-fold dilutions. Suitable buffering and poising of the dilution fluid by the addition of a low concentration of regular medium is desirable. Additional advantages in the use of 0.1% medium for dilution are that the dilution bottles may be pretested for sterility prior to use, and also, since the bottles foam slightly when shaken, errors during the dilution process are avoided.

Most probable number technique

Five tubes containing approximately 10 ml. of regular medium (penicillin or streptomycin may be added to inhibit bacterial growth, if technique is not rigidly aseptic) were then inoculated with one ml. from each of the above-mentioned dilutions. After a suitable incubation period (96 hours), the tubes were checked for positive growth.

The most probable number of organisms in the original culture was found by reference to the data shown in Table I. The number of positive tubes (from each

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[1] Post-doctoral Fellow in Cancer Research of the Damon Runyon Memorial Fund.

Table I

Most probable number per milliliter of sample, planting 5 portions in each of 3 dilutions in geometric series. (Modified from Hoskins, 1934)

(1) x			(2)	(1) x			(2)	(1) x			(2)	(1) x			(2)	(1) x			(2)	(1) x			(2)
1	.1	.01		1	.1	.01		1	.1	.01		1	.1	.01		1	.1	.01		1	.1	.01	
0	0	0		1	0	0	2	2	0	0	4.5	3	0	0	7.8	4	0	0	13	5	0	0	23
0	0	1	1.8	1	0	1	4	2	0	1	6.8	3	0	1	11	4	0	1	17	5	0	1	31
0	0	2	3.6	1	0	2	6	2	0	2	9.1	3	0	2	13	4	0	2	21	5	0	2	43
0	0	3	5.4	1	0	3	8	2	0	3	12	3	0	3	16	4	0	3	25	5	0	3	58
0	0	4	7.2	1	0	4	10	2	0	4	14	3	0	4	20	4	0	4	30	5	0	4	76
0	0	5	9	1	0	5	12	2	0	5	16	3	0	5	23	4	0	5	36	5	0	5	95
0	1	0	1.8	1	1	0	4	2	1	0	6.8	3	1	0	11	4	1	0	17	5	1	0	33
0	1	1	3.6	1	1	1	6.1	2	1	1	9.2	3	1	1	14	4	1	1	21	5	1	1	46
0	1	2	5.5	1	1	2	8.1	2	1	2	12	3	1	2	17	4	1	2	26	5	1	2	64
0	1	3	7.3	1	1	3	10	2	1	3	14	3	1	3	20	4	1	3	31	5	1	3	84
0	1	4	9.1	1	1	4	12	2	1	4	17	3	1	4	23	4	1	4	36	5	1	4	110
0	1	5	11	1	1	5	14	2	1	5	19	3	1	5	27	4	1	5	42	5	1	5	130
0	2	0	3.7	1	2	0	6.1	2	2	0	9.3	3	2	0	14	4	2	0	22	5	2	0	49
0	2	1	5.5	1	2	1	8.2	2	2	1	12	3	2	1	17	4	2	1	26	5	2	1	70
0	2	2	7.4	1	2	2	10	2	2	2	14	3	2	2	20	4	2	2	32	5	2	2	95
0	2	3	9.2	1	2	3	12	2	2	3	17	3	2	3	24	4	2	3	38	5	2	3	120
0	2	4	11	1	2	4	15	2	2	4	19	3	2	4	27	4	2	4	44	5	2	4	150
0	2	5	13	1	2	5	17	2	2	5	22	3	2	5	31	4	2	5	50	5	2	5	180
0	3	0	5.6	1	3	0	8.3	2	3	0	12	3	3	0	17	4	3	0	27	5	3	0	79
0	3	1	7.4	1	3	1	10	2	3	1	14	3	3	1	21	4	3	1	33	5	3	1	110
0	3	2	9.3	1	3	2	13	2	3	2	17	3	3	2	24	4	3	2	39	5	3	2	140
0	3	3	11	1	3	3	15	2	3	3	20	3	3	3	28	4	3	3	45	5	3	3	180
0	3	4	13	1	3	4	17	2	3	4	22	3	3	4	31	4	3	4	52	5	3	4	210
0	3	5	15	1	3	5	19	2	3	5	25	3	3	5	35	4	3	5	59	5	3	5	250
0	4	0	7.5	1	4	0	11	2	4	0	15	3	4	0	21	4	4	0	34	5	4	0	130
0	4	1	9.4	1	4	1	13	2	4	1	17	3	4	1	24	4	4	1	40	5	4	1	170
0	4	2	11	1	4	2	15	2	4	2	20	3	4	2	28	4	4	2	47	5	4	2	220
0	4	3	13	1	4	3	17	2	4	3	23	3	4	3	32	4	4	3	54	5	4	3	280
0	4	4	15	1	4	4	19	2	4	4	25	3	4	4	36	4	4	4	62	5	4	4	350
0	4	5	17	1	4	5	22	2	4	5	28	3	4	5	40	4	4	5	69	5	4	5	430
0	5	0	9.4	1	5	0	13	2	5	0	17	3	5	0	25	4	5	0	41	5	5	0	240
0	5	1	11	1	5	1	15	2	5	1	20	3	5	1	29	4	5	1	48	5	5	1	350
0	5	2	13	1	5	2	17	2	5	2	23	3	5	2	32	4	5	2	56	5	5	2	540
0	5	3	15	1	5	3	19	2	5	3	26	3	5	3	37	4	5	3	64	5	5	3	920
0	5	4	17	1	5	4	22	2	5	4	29	3	5	4	41	4	5	4	72	5	5	4	1600
0	5	5	19	1	5	5	24	2	5	5	32	3	5	5	45	4	5	5	81	–	–	–	——

(1) Significant number: positive tubes* with dilutions 1x, 0.1x and 0.01x.
(2) Most probable number per milliliter.

* The dilution "x," used in the tubes, representing the first digit in the significant number, is corrected as follows:

Where x is a dilution of	*Multiply MPN value by*
1:10	1
1:100	10
1:1000	100
etc.	

group of five) in the entire series of geometric dilutions may be represented as a multi-digit number, *e.g.,* 5 5 5 4 3 1, in which the first digit represents the number of positive tubes from the 1 : 10 dilution, and the last number from the 1 : 1,000,000 solution. The number to be used for reference to the table, *i.e.*, the significant number, is 5 4 3 1. The first digit of the significant number represents the number of tubes of the highest dilution from which all were positive, or the lowest dilution used, in the event less than 5 are positive. Since the significant number must consist of three digits, any fourth digit is added to the third, and the example given above now becomes 5 4 4. Reference to column 1 of the table indicates a most probable number of 350. Since the dilution for the first digit of the significant number was 1 : 1000, the most probable number is multiplied by the correction factor indicated in the table. In the illustration given, we obtain 35,000 organisms per milliliter as the most probable number.

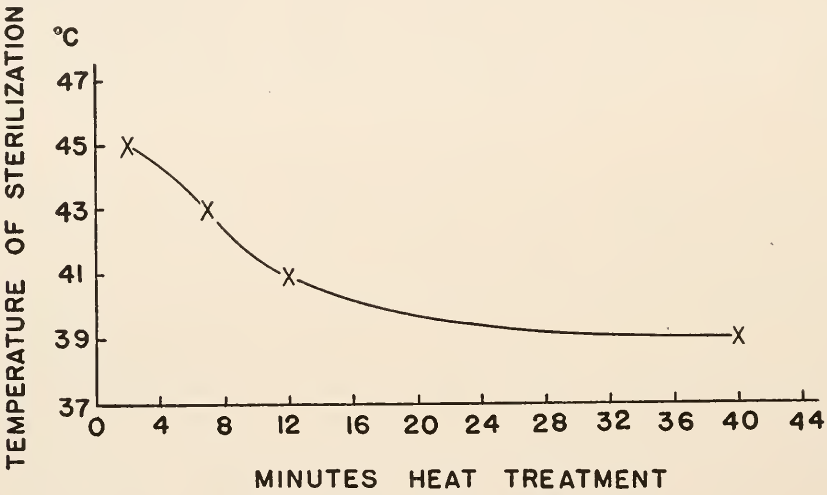

FIGURE 1. Thermal death time for *Tetrahymena pyriformis* WB.

Correlations were made between direct microscopic counts (Hall, Johnson and Loefer, 1935) and most probable number determinations. Average results of either did not vary by more than 5% when replicate determinations were made. The slightly higher count observed consistently in direct counts is readily explicable since any culture contains a certain number of non-viable organisms which will not be detected in the most probable number determination.

EXPERIMENTAL

Viable survivors at specified time intervals were determined following treatment of cultures at 39, 41, 43 and 45° C. In addition, thermal death time at each temperature was determined and these data are presented in Figure 1. The sharp increase in the thermal biological effect as the temperature is raised is due to the high Q_{10} of protein denaturation. At 37° C., several hours were required to obtain a sterile culture.

Although survivor curves were obtained at each of the temperatures tested, those following treatment at 39 and 45° C. are presented as being typical (Fig. 2). As opposed to the case following treatment with ultraviolet radiation (Mefferd and Loefer, 1952), there was a close correlation between loss of motility and loss of viability. In all cases there is a period of relatively slow death rate preceding the period of most rapid killing. A part of this lag undoubtedly is a result of the time required to raise individual cells to the temperature of the water bath, but this explains only a part of the effect. The most logical explanation remains that given by Rahn (1931) which assumes that there is more than one molecule which must be destroyed in order to cause death of the cell. A possibility which must be considered is that the protozoan population varies widely in the thermal resistance of its members (Jahn, 1933). When large inocula are employed, it is not difficult to select a population of cells with markedly enhanced viability at high temperatures (Mefferd and Campbell, 1952). The authors have, by such techniques, obtained strains of *T. pyriformis* which are capable of growth at 37° C. and even higher. However, in the usual population of a few million cells, the portion which is heat resistant would only negligibly affect the survival curve.

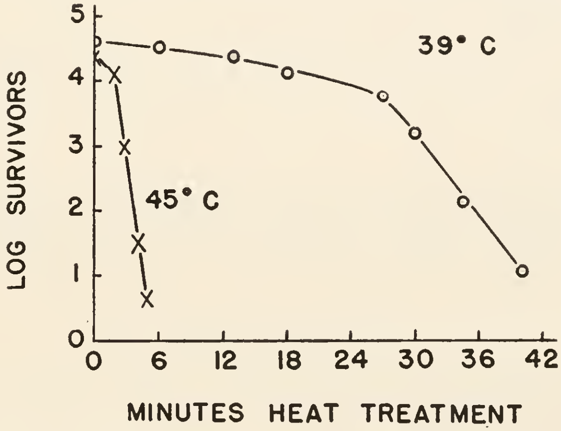

FIGURE 2. Survivor curves for cultures of *Tetrahymena pyriformis* WB subjected to 39 and 45° C.

Our data agree generally with those reported by Jahn (1933) for *Euglena gracilis*, and the slight difference may be explained by two major modifications in our technique which varied from his, *viz.*, the introduction of a very small volume of concentrated organisms into a large volume of pre-heated medium, and the quantitative determination of viable cells utilizing a technique (most probable number) which is dependent only upon whether or not an aliquot contains one or more viable cells. This eliminates the many factors which may influence methods based upon comparative growth rates, such as unequalized population densities and the difference in nutritional supplementation of the media between a culture in which no organisms are killed and another in which many have been killed and lysed. The importance of either of these factors is evident.

It is important for the successful application of the technique that incubation of tubes be continued for an extended period of as many as six days or so. In each series there are tubes which contain as few as one organism. Starting from this small inoculum, considerable time is required to achieve a detectable number of cells in the tube. A small quantity of a suitable antibiotic in the dilution and growth media is a distinct advantage in inhibiting chance bacterial contaminants.

Summary

1. Utilizing the most probable number technique, thermal death time and viable survivor curves over a series of temperatures (39, 41, 43 and 45° C.) were determined for Tetrahymena.

2. During the early stages of heat treatment, an increasing death rate was observed in the case of each temperature, but the rate became constant (logarithmic) after a time.

3. At the higher temperatures (45° C.), the logarithmic rate was achieved in a very short time.

4. Use of the most probable number technique eliminates several of the criticisms which have been made of previous work upon the thermal sensitivity of protozoa, and enables a rapid and reliable estimation of viable cells following heat treatment. The advantages and limitations of the method as applied to protozoa are discussed.

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FACTORS INFLUENCING METAMORPHOSIS OF BUGULA LARVAE

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A review of the literature on experimentally induced metamorphosis in invertebrates and in sessile tunicates suggests the possibility that essentially similar mechanisms may be involved in animals of widely different phyla. As in parthenogenesis, metamorphosis can be induced experimentally by a wide variety of conditions and chemical agents, generally lacking any semblance of specificity; and most of these seem to be unfavorable to larval life. Huxley (1928) effected metamorphosis in Echinus plutei by treating them with very dilute concentrations of $HgCl_2$ ($M/2{,}000{,}000$), and Runnström (1917) found that starvation of the larvae, over-ripeness of the eggs from which they developed and very dilute solutions of $ZnSO_4$ in sea water would cause dedifferentiation in Echinus larvae. Grave (1935) found that ascidian metamorphosis can be accelerated by many agents including CO_2-free sea water, lactic acid, precipitates formed when NaOH, KOH or $BaCl_2$ are added to sea water, whole pituitary gland, tissue extracts of ascidian mantle and endostyle and of the foot and mantle of snails; these extracts are highly specific in nature. Other promoters of ascidian metamorphosis include: copper (Grave, 1941; Bertholf and Mast, 1944; Glaser and Anslow, 1949), concentrated sea water (Bertholf and Mast, 1944), thyroid extract (Weiss, 1928) and whole gland (Grave, 1935), methylene blue (Bertholf, 1945; Zhinkin, 1938), neutral red (Bradway, 1936; Zhinkin, 1938), brilliant cresyl blue (Zhinkin, 1938), Janus green (Bertholf and Mast, 1944), a brief exposure to distilled water (Bertholf, 1945), inorganic iodine but not thyroxine (Bradway, 1936), "conditioned sea water" (Grave and Nicoll, 1936; Grave, 1944; Bertholf and Mast, 1944) and isotonic NaCl (Bradway, 1936). (Grave, 1935, had found that the addition of sodium and potassium ions to sea water had no effect on metamorphosis; Bradway, however, used a different technique with an optimum amount of exposure and used isotonic NaCl instead of sea water to which the ions had been added.) Metamorphosis can be induced in the larva of the oyster, *Ostrea virginica,* by copper (Prytherch, 1934; Armstrong and Miall, 1945) and by a certain optimum of salinity (Prytherch, 1934); but neither the cations nor anions of sea water nor the metal carbonates of Fe, Al, Ag, Ni, Ba, Pb and Sn have any effect according to Prytherch (1934). Copper, hypertonic sea water and heat accelerate metamorphosis in the bryozoan, *Bugula flabellata* (Lynch, 1949a).

Since fluids in which proteolytic digestion has occurred bring about a high concentration of neutral red from very dilute solutions, whereas coelomic fluids do not take the stain, Koehring (1930, 1931) proposed that the azo radical of the dye combines with and activates proteolytic enzymes and that neutral red staining can be used as an index of the site and intensity of enzyme activity. She found the dye effective in causing parthenogenesis in starfish eggs. Neutral red in concentrations of 1 : 50,000 parts (Bradway, 1936) and of 1 : 200,000–1 : 800,000 parts in sea water (Zhinkin, 1938) accelerates metamorphosis in ascidian larvae, presumably by activating autolytic enzymes involved in resorption of the tail. Zhinkin (1938) found methylene blue also effective in approximately the same concentrations. Since some agents have the common effect of inducing precocious fixation in animals of widely different phyla, it was thought that the two vital dyes might accelerate metamorphosis in *Bugula* larvae.

The present paper concerns a re-investigation of the role played by the ions of sea water under more rigidly controlled conditions of light, osmotic pressure and the pH of the medium than in former experiments, and of the effects of media containing only three or two ions and combinations of these with vital dyes; the work was undertaken since it was considered that certain factors may act antagonistically to accelerators of metamorphosis whereas others may synergize their effects. This supposition was verified, sometimes in a striking manner. The larvae were secured by means already described (Lynch, 1947) and the methods used will be presented in the separate sections. Casters and syracuse dishes were used for most observations with a binocular microscope and these were kept covered to prevent shifting of the pH; when controls were used they were kept at the same number of foot candles of illumination (Weston photometer) as the experimental dishes, since light has an accelerating effect on metamorphosis.

RESULTS

An excess of the chlorides of Na, K, Ca and Mg in sea water. General observations on the effects of concentrating these chlorides in sea water have already been published (Lynch, 1947, 1949a). During the summers of 1951 and 1952 experiments on the effects of an excess of each of the four chlorides were carried out by using only isotonic solutions in sea water, in proportions of 80/20, 50/50 and 20/80 cc. For magnesium and potassium the results were essentially the same as those reported (Lynch, 1949a). Metamorphosis was inhibited at a pH of 7.1, that of the original mixtures, and at 8.0 (raised by NaOH in magnesium mixtures and by

TABLE I

The effects of an excess of NaCl on the motility of larvae

Larvae in 80 cc. isotonic NaCl/20 cc. sea water pH = 8.1 (NaOH)				Larvae in normal sea water on the same day			
No. of larvae	No. active in hours			No. of larvae	No. active in hours		
	5	12	24		5	12	24
70	50	0	0	34	16	5	1
115	47	3	0	37	22	2	1
48	16	0	0	59	24	7	1
8	5	0	0	29	14	4	1
12	8	0	0	12	5	4	0
—		—		—		—	
253		3 = 1.1%		171		22 = 12.2%	

KOH in those containing an excess of potassium). The prolonged swimming, formerly observed in the magnesium mixtures, was evidently due to the hydronium ion, since it does not occur at a pH of 8.0 and experiments with sea water acidulated by HCl have shown a considerable lengthening of the natant phase. Since the almost immediate metamorphosis observed in the 50/50 mixtures of 1 N NaCl and $CaCl_2$ could have been effected by the hypertonicity of these solutions, especially the former, the experiments were repeated with isotonic NaCl and $CaCl_2$. A high osmotic pressure was undoubtedly the cause of accelerated metamorphosis in the case of sodium chloride, for proportions of 80 cc. of isotonic NaCl/20 cc. of sea water did not hasten setting; in fact, metamorphosis did not occur in some experiments. In general, only larvae that attached to the surface or to the photonegative side of the dishes containing an excess of NaCl metamorphosed normally. The natatory period, however, was abbreviated as reported for the normal solutions (see Table I). On the other hand 80 cc. of isotonic $CaCl_2$/20 cc. of sea water gave results essentially identical with those described for normal solutions, but metamorphosis occurred almost immediately only when the pH had been raised to 8.0 ($CaCO_3$, NaOH, KOH and borate buffers gave similar results). The reverse proportions, however (20 cc. of $CaCl_2$/80 cc. of sea water), failed to show any statistically significant difference between the behavior of larvae in these media and that of the controls. The cause of the observed prolongation of the natatory period in solutions having the same proportions of sea water and 1 N $CaCl_2$ remains obscure. It seems unlikely that it was mere coincidence, for larvae are rarely active at 24 hours in sea water (*cf.*, Table II), unless the pH is lowered to about 6.5, and experiments with isotonic solutions gave no indication of such a drop in pH. (Table III shows that many larvae are active at 24 hours in these solutions at a low pH.) These studies have also shown that hypertonic solutions ac-

TABLE II

The normal duration of the natatory phase in sea water

No. of larvae	No. inactive in hrs.			
	4	8	12	24
73	71	—	73 (100%)	73
39	28	—	33 (87%)	39
56	21	—	35,(62%)	56
86	7	28	38 (44%)	73
138	84	102	123 (90%)	129
109	4	14	66 (60%)	109
34	—	—	29 (85%)	33
37	—	—	35 (94%)	36
59	—	—	51 (86)	58
29	—	23	25 (86)	28
12	—	3	8 (66)	12
14	—	10	13 (93)	14
686			529	

Average number inactive at 12 hrs. = 77%. Sigma = 16%.

celerate metamorphosis, regardless of whether the osmotic pressure is raised by ions of salts or by molecules of sugar. In former experiments with solutions made hypertonic by adding sucrose to sea water, the osmotic pressure had not been raised sufficiently high to give accelerating effects. (Hastening of setting does not occur if the freezing point depression is much below 2.73.)

TABLE III

The natatory period of larvae in 80 cc. of sea water and 20 cc. $CaCl_2$ at pH = 6.4

Experimental medium		Controls (same days; pH = 6.4)	
No. larvae	No. active at 24 hrs.	No. larvae	No. active at 24 hrs.
84	17	88	2
81	6	44	1
76	6	89	5
185	25	58	0
145	41	64	4
101	25	69	0
41	4	12	2
31	6	16	2
744	130 = 17.4%	440	16 = 3.6%

Three-ion combinations. These mixtures were made by omitting one of the ions from the following proportions used by LeFevre (1948) in making artificial sea water from isotonic solutions: 500 cc. of NaCl, 10 cc. of KCl, 15 cc. of $CaCl_2$, 2 cc. of $NaHCO_3$, 40 cc. of $MgCl_2$ and 15 cc. of $MgSO_4$. Van't Hoff's (beta) solutions, however, were used for calcium-free media and $MgSO_4$ was omitted in all solutions containing calcium (because of precipitation) and its place was taken by 15 cc. of $MgCl_2$. Experiments were performed with solutions in which the missing ions were replaced by an equal volume of isotonic sucrose and with mixtures without sugar but having the three ions in the same ratio as in artificial sea water. The presence or absence of sucrose seemed to have no effect, although it was found that metamorphosis was inhibited in solutions containing 80 cc. of sucrose/20 cc. of sea water at a pH of 7.8 (glycine buffer). This procedure of making three-ion media is open to the objections summarized by Heilbrunn (1943, p. 455) and the absence of sulphate may have had an independent effect. (For the effects of an absence of sulphate on echinoderm animal-halves treated with lithium, see Needham, 1950, p. 492.) Larvae were placed for a minute or two in 15 cc. of the three-ion medium before being transferred to another dish of the same solution. Generally three or four transfers were made to minimize contamination during seeding with larvae. Nevertheless, the term "ion-deficient media" is preferable to "ion-free" since a slight amount of contamination is unavoidable. The pH was raised to 7.8–8.0 by NaOH and KOH in all experiments.

In all except Mg-free solutions metamorphosis failed to occur. Results were quite similar in Na-free, Ca-free and K-free media. In all of these the second stage of metamorphosis, migration of tissue from the pallial furrow and subsequent pushing of the ciliated covering towards the basal end of the larvae, could be observed; but the first stage, eversion of the internal sac, failed to occur. This may not be quite true of larvae in Na-free media, for the internal sac protruded from the organisms while swimming but failed to attach them to the substrate. A great elongation of the apical end accompanied by a migration of ciliated covering towards the basal region caused the long axis of the larvae to be lengthened and a prominent bulge to appear in the middle where the ciliated tissue had accumulated in the Na-free media. After an hour and a half the larvae became spherical again and the cilia seemed to disintegrate eventually. Organisms in Ca-free van't Hoff's solution failed to show any protrusion of the internal sac and eventually shed their outer ciliated coverings into the surrounding medium exactly like those placed in an excess of $MgCl_2$ (Lynch, 1949a). The larvae exhibited neither the usual light reactions nor the back-and-forth swimming of the controls. Highly concentrated in the center of the dish, these larvae confined their movements to narrow circumscribed areas, consisting of either circular counterclockwise swimming or rotation about a fixed point. By 6–8 hours these movements had ceased but ciliary action continued. (The latter was most prolonged at a pH of 6.3.) By 24 hours cytolysis was pronounced in larvae left in this medium. Motility was much better preserved in Ca-free than in Na-free media, for in the latter there was scarcely any movement at 1½ hours. In K-free media the behavior was essentially like that described above, except that adhesive fluid was gradually shed from the internal sac and congealed behind the larvae while they were still swimming. (This phenomenon is not at all uncommon in experimental media, for it occurs in sea water when the pH is lowered to 7.0 by McIlvaine buffer and in other cases to be described.) Again, in K-free solutions the behavior of the larvae strikingly resembled that of organisms placed in an excess of magnesium chloride.

Metamorphosis occurred only in Mg-free media within a pH range of 7.4 to 8.0 (raised by NaOH in the latter). Setting was considerably accelerated, having been completed in nearly all larvae within a period of 30 minutes to 3 hours. (The normal duration of the natatory period of larvae in sea water is variable, sometimes occurring within two hours and occasionally enduring in a few larvae for 24 hours: *cf.*, Table II. Several trials have shown that by 12 hours 77% ± 5 have metamorphosed.) When metamorphosed larvae were transferred from the Mg-free media to sea water after 3 hours they reached the polypide stage by 24 hours; those left in the original solutions elongated somewhat but did not reach the condition at which stolons are organized into tripod-shaped structures for anchorage, which usually occurs about 8 hours after metamorphosis. All adhered rigidly to the substrate. The first reaction of larvae placed in Mg-free media was one of complete quiescence; then natatory movements were gradually resumed. Many of the organisms emitted small amounts of adhesive material from the internal sac shortly after contact with solutions at a pH of 7.4, that of the original mixture. A deficiency or absence of magnesium had an effect opposite to that of an excess of the ion.

Two-ion combinations. Normal motility of bryozoan larvae can be maintained for an amazingly long time when only two ions are present. (1) In 20 cc. of isotonic NaCl/10 cc. of $MgCl_2$ (pH raised to 8.0 by NaOH) the larvae remained motile for 6–8 hours after three transfers. Again the ciliated covering migrated towards the basal region of the larvae and these organisms lost their reaction to light, gathering in enormous numbers in the center of the dish. In fact, an unusual protrusion of the apical organ and migration of ciliated covering towards the basal end, or at least the equatorial region, of the larvae seems to be a characteristic reaction when any one of the four ions of sea water is missing from the media. (2) When the proportions were reversed (20 cc. of $MgCl_2$/10 cc. of NaCl at a pH of 8.0) motility was maintained for as long as 10 hours and typical magnesium effects were observed: absence of light reactions and emission of tissue from the pallial furrow. (3) Similar phenomena occurred in 15 cc. of $CaCl_2$/55 cc. of $MgCl_2$ and motility endured for at least 4 hours after three transfers. This is surprising in view of the fact that natatory movements are extremely feeble by 1½ hours in three-ion Na-free solutions. Evidently the absence of potassium favors ciliary action, for it is more injurious to the cilia of the larvae than any other salt except copper when an excess is added to sea water. Likewise magnesium seems to be necessary for natatory movements of long duration, for in Mg-free three-ion combinations only feeble movements persisted for three hours; in solutions of 20 cc. NaCl/10 cc. of $CaCl_2$ (pH = 8.0 by NaOH) the larvae became immobilized almost immediately and began rotating about a fixed point, a movement that immediately precedes metamorphosis under normal conditions, by means of their vibratile flagella. (The latter form a tuft of long hair-like structures at the apical end of the median furrow or lateral groove; movements of these flagella persist long after ciliary action has ceased.) Great care must be taken to separate ionic effects from those due to the pH of the medium, since larvae in sea water acidulated to a pH of 5.8–6.0 (HCl) may continue swimming for 54 hours or longer, even though the pH will have shifted to 7.0 during 24 hours. Metamorphosis did not occur in any of the two-ion combinations after two transfers, although an atypical kind of setting was observed in 20 cc. of NaCl/10 cc. of $CaCl_2$. Although the larvae did elongate somewhat when removed to sea water after a short exposure to this medium, repeated observations could not determine whether this was a true metamorphosis or not. It is difficult to distinguish the chaotic condition of metamorphosis from rapid cytolysis that generally occurs under these conditions. Even the small amount of sea water added during seeding allowed normal setting in 20 cc. of $MgCl_2$/10 cc. of NaCl; larvae on the surface formed zooids with tripod-like stolons for anchorage. (It is interesting to note in passing that this is characteristic of *B. flabellata,* whereas *B. turrita* always develops four symmetrically arranged stolons, each branching dichotomously at its distal end into two branches.)

Neutral red in sea water. In the following experiments one drop of 0.1% aqueous solution of neutral red was added to 10, 30, 50, 100, 150 and 200 cc. of sea water (range of concentration = 0.001–0.00005%; 1 : 100,000–1 : 2,000,000 parts). The pH of the highest concentration was 7.8–8.0 and the freezing point depression was 1.71. Both *B. turrita* and *B. flabellata* were employed during the summer of 1951, but only the latter in 1952; the controls and the experimental organisms were always of the same species and dishes containing them were placed in diffuse light of 75 foot-candles. Temperatures varied from 25–29° C. Larvae were transferred once from a test solution to another of equal concentration to minimize the dilution which occurred during seeding.

Two striking effects were caused by the neutral red media. First, the photonegative response, which generally takes place in sea water at two–three hours, had set in by an hour in all solutions; and in most cases the change from positive to negative phototropism began within ten minutes after placement of the larvae. These organisms were also more intensely negative than the controls and frequently attached *en masse* at a spot on the periphery of the dish farthest from the source of light. Secondly, metamorphosis was accelerated in solutions containing one drop/10, 50 and 100 cc. of sea water (concentrations = 0.001–0.0001%), but dilutions greater than 0.0001% had no appreciable effect. The degree of acceleration appeared to be somewhat roughly proportional to the concentration; except in media containing one drop/10 cc., the duration of the natant phase varied with that of the controls, being shortest on days when the latter metamorphosed more quickly. In solutions containing one drop/10 cc. of sea water, the free-swimming period was about an hour with very little deviation on either side. In the other solutions found to be effective the extreme duration of the natatory period was four hours; at this time only a few larvae were still active, whereas the majority of the controls were motile at 12 hours.

Subsequent development depended upon whether the larvae were left in the neutral red solution or transferred to sea water after metamorphosis. The results of various concentrations may be summarized as follows: (1) Larvae in the most concentrated solution (1/10) had an elongation equal to that of the controls by 24 hours, but actual development was better when the larvae were transferred to sea water after metamorphosis had been induced precociously, for in the latter case differentiation sometimes exceeded that of the controls. In one experiment in which larvae were allowed to remain in the neutral red solution, about ⅔ had become decidedly stained and at 24 hours these had formed normal zooids as well advanced as the controls except that polypide formation was somewhat inferior; the other ⅓ were free from dye and had not metamorphosed. (2) In solutions of one drop/50 cc. of sea water development was inferior to that of the controls in three out of four experiments in which the larvae remained in the neutral red solution for 24 hours; differentiation was normal, however, when the organisms were removed to sea water after metamorphosis. In at least some cases of poor zooid formation the effect can be attributed to a loss of fluid from the internal sac while the larvae were still swimming; this fluid congealed to form threads which resembled those that had been observed when organisms were placed in sea water maintained at a pH of 7.0 by potassium phosphate McIlvaine buffers, except that the threads formed a network in the latter. Since the holdfast material forms the zooecial wall of the zooid, its deficiency would result in poor development. (For the contribution of larval parts to the adult organism the monograph of Corrêa (1948) on the embryology of *Bugula flabellata* should be consulted.) (3) In dilutions as great as one drop/100 cc. development was equal to that of the controls even when the larvae were not transferred to sea water. (4) In solutions found to be ineffective in appreciably accelerating metamorphosis, development was variable (as it often was in the controls), and in general it seemed to be no different from that of larvae in sea water.

Staining reactions of neutral red. The apical half of the pyriform larvae (containing the crown of rigid cilia) stained very lightly, whereas the basal half (containing the internal sac) stained heavily, the dye being concentrated in definite spots causing the larvae to have a mottled appearance. Staining was generally pronounced within 20–30 minutes after immersion in the most concentrated media (one drop of neutral red/10 cc. of sea water and one drop/30 cc.). During the process of metamorphosis, material from the pallial furrow (lacking cilia) did not take the dye; and as this material migrated slowly to the basal end of the larvae, the dye gradually became concentrated in granular spots beneath it in this region. The ciliated covering, which is gradually pushed inside the metamorphosing larva by advancing material from the pallial furrow, did not take the stain. This could be determined under abnormal conditions (Ca-free solutions) in which the ciliated covering was shed into the surrounding medium. Holdfast material was likewise largely colorless except for a few granules. After metamorphosis had been completed, the zooecial wall of the first polypide was almost free from stain except for a few red granules scattered here and there but especially concentrated at the distal end where the lophophore would form later. The former body of the larva, located about a third of the distance between the attached end and the free lophophore region, was deeply stained. In later development the polypide, containing the digestive tract, was deeply stained within the zooecial wall. Some of the dye went into the bud for the second zooid of the colony, again concentrating at the tip where the lophophore would form. Buds for the third zooid generally remained free from stain. The tentacles as well as the stolons, except at the distal ends of the latter, were colorless.

Neutral red in three-ion combinations. These media were made as described above and one drop of neutral red/10 cc. of the three-ion media was added. Except for a decrease in motility the behavior of larvae in these media was essentially the same as in these solutions without dye. Neutral red, although a powerful accelerator of metamorphosis, was ineffective in bringing about this phase of the life cycle of the larvae. In fact, there have been no cases encountered so far in which neutral red has effected metamorphosis in solutions that inhibit the process. (This is true also of a pH below 6.0.) The only significant differences in the K-free, Ca-free and Na-free media were: (1) In K-free solutions minute amounts of adhesive material were lost and as the ciliated covering was shed, it became entrapped in this fluid; this did not occur in Ca-free and Na-free solutions, for in the former the ciliated cells were shed into the medium and in Na-free there was no noticeable loss of ciliated covering. (2) Larvae first placed in the ion-free media and then removed to sea water within an hour showed obvious elongation only in the case of Na-free media; there was a very slight elongation of larvae placed in K-free media, but the organisms were only one-fourth normal size (probably because of the loss of adhesive fluid). Larvae in these media failed to show the violent photonegative response of the controls (the same concentration of dye and sea water), generally being quite indifferent to light; in general, the organisms behaved remarkably like those in sea water containing an excess of magnesium chloride. The absence of any one of the ions, except perhaps Na, definitely inhibited metamorphosis, probably by accentuating the anaesthetizing effects of magnesium.

Larvae in Mg-free media exhibited the most striking effects, for a deficiency of the ion accentuated the accelerating properties of neutral red, metamorphosis taking place within 15–30 minutes. Organisms that remained in this solution had slightly elongated by 24 hours, whereas those removed to sea water developed normally but at a somewhat retarded rate, having developed polypides only by 38 hours. (Polypides are normally recognizable before 24 hours.) All these larvae attached rigidly to the substrate. In view of the inhibiting effects of magnesium, these results might have been anticipated.

Neutral red in sea water at reduced temperatures. In previous experiments it had been observed that cold sea water inhibited metamorphosis of the larvae of *B. neritina* (Lynch, 1947). If neutral red accelerates metamorphosis by activating larval enzymes, a reduction of temperature should reduce the effect. To verify this hypothesis two test solutions were made by adding one drop of neutral red/10 cc. of sea water; the temperature of one of these was maintained at 4° C. and the other was kept at 12° C. The motility of larvae immersed in these media was greatly reduced by half an hour and the organisms all settled to the bottom exhibiting neither positive nor negative reactions to light. Metamorphosis did not occur at the usual time for larvae in this concentration of neutral red, for only a few had begun the process by an hour and fifteen minutes and these did not attach rigidly as they did at room temperature. The second stender dish (12° C.) was allowed to warm at this time, and by an hour and forty-five minutes only 20% had metamorphosed; these had their internal sacs everted upwards. By four hours only 50% had metamorphosed and these organisms showed no elongation by 24 hours; the controls (larvae in the same concentration of neutral red and sea water at room temperature) had metamorphosed by an hour. Cooling the medium, therefore, inhibited metamorphosis even after the solution came to room temperature.

TABLE IV

The effects of concentration of neutral red on the duration of the natatory period

I. Larvae in one drop of 0.1% aqueous soln. of neutral red/10 cc. of sea water				II. Larvae in one drop of 0.1% aqueous soln. of neutral red/30 cc. of sea water			
No. larvae	Number unmetamorphosed in hours			No. larvae	Number unmetamorphosed in hours		
	1	2	3		1	2	3
19	2	2	0	45	19	4	1
27	1	0	0	75	64	6	0
30	2	0	0	91	62	4	1
27	3	0	0	19	13	1	1
25	1	0	0	53	39	25	21
45	9	0	0	42	20	6	1
84	9	9	0	298	188	43	4
65	19	6	0	303	243	19	1
42	13	2	0	42	14	8	1
85	1	0	0	30	13	10	3
52	0	0	0	27	14	10	3
22	7	0	0	16	15	8	7
23	3	0	0				
—	—			—	—		
547	70 = 12.8%			1041	704 = 67.2%		

Neutral red in isotonic solutions. These media consisted of one drop of neutral red/10 cc. of isotonic solutions of the four chlorides. Metamorphosis was not observed in any of them and motility was greatly reduced, never enduring for more than half an hour. Calcium chloride had the most deleterious effect, causing the cilia to dissolve and bringing about cytolysis of the larvae to a marked degree. In the potassium solution ciliated tissue was shed just as it was in sea water containing an excess of this ion (Lynch, 1949a). In the sodium chloride solution the larval covering also disintegrated instead of being pushed inside by tissue from the pallial furrow which had begun to migrate towards the basal end. Magnesium chloride merely anaesthetized the larvae causing the cilia to stand out like a circlet surrounding them. These results were similar to those obtained by using isotonic solutions without neutral red. It is interesting to note that Bradway (1936) had found isotonic calcium chloride to be the most deleterious of all the chlorides in its effect on ascidian larvae; magnesium chloride was relatively non-toxic and inhibited metamorphosis.

Methylene blue in sea water. Methylene blue, though much less effective in accelerating metamorphosis than neutral red, gave results similar to those just described. Only two concentrations were used, one drop of 0.1% aqueous solution/10 cc. and 50 cc. of sea water. The pH was 7.6 and the F.P.D. was 1.71. Motility was considerably greater than in similar concentrations of neutral red and the photo-negative response not only occurred later but was also less pronounced. Although the blue dye was not absorbed as readily nor as extensively as neutral red, the staining reactions were similar, the apical end being almost colorless; whether this was caused by a failure of this region to absorb the dye or by a reduction process is not known. More larvae were geo-negative in the methylene blue media than in those containing neutral red. Except for larvae transferred to sea water after metamorphosis, zooid formation was rather poor in the more concentrated solution; those that attached to the surface, however, developed normally even when they were not transferred to sea water, but geo-negative settings did not.

Discussion

Several explanations of the *modus operandi* of metamorphosis have been offered in the past by various investigators. Thirty years ago Huxley (1922) suggested that unfavorable environmental conditions can bring about the changes, generally cataclysmic, that reduce a free-swimming larva to a state of morphological chaos followed by a constructive phase of differentiation and growth. After comparing similarities between normal metamorphosis in Echinus plutei and dedifferentiation in hydroids and in the ascidians, *Clavelina* and *Perophora*, he concluded that the two processes are essentially similar and predicted that toxic agents would induce precocious metamorphosis in echinoderm larvae.

Certainly copper, $HgCl_2$, $ZnSO_4$, distilled water, isotonic salt solutions, starvation and vital dyes would seem to affect larvae adversely. (For the relative toxicities of neutral red, methylene blue and Janus green, see Child and Rulon, 1936.) Although there is much logic in the proposition of Huxley (1928) that Echinus larvae metamorphose when their increasing weight causes them to sink to the bottom away from the more favorable conditions of food and oxygen at the surface (and this idea would be applicable to ascidians, which become geo-positive just before setting), the same explanation cannot be used for bryozoan larvae that frequently attach to the surface. Although the internal environment might become unfavorable because of the accumulation of toxic products of metabolism or starvation (since an alimentary canal is absent), the external environment is apparently unchanged just before setting. The hypothesis that an unfavorable environment causes metamorphosis is an unsatisfying one from a chemical viewpoint, since it does not explain why metamorphosis occurs under adverse conditions.

At first sight the hypothesis that larval enzymes are involved in metamorphosis seems to have much in its favor. The observations already presented, as well as those of Bradway (1936) and Zhinkin (1938), on the effects of vital dyes in accelerating metamorphosis would seem to indicate that a stimulation of proteolytic enzymes is somehow involved. On the other hand, Glaser and Anslow (1949) proposed as a tentative hypothesis that copper inactivation of larval enzymes is the "key to morphological disintegration" characteristic of the disruptive phase of ascidian metamorphosis. How, then, can two viewpoints, one of stimulation of larval enzymes and the other of inactivation, be reconciled? It may be that some agents, such as copper, can inactivate certain enzymes, perhaps succinic-dehydrogenase systems, that are necessary for larval life; others may stimulate autolysis. The same net result—larval dedifferentiation—could presumably be attained by stimulating proteolytic enzymes or by inactivating larval oxidation-reduction systems. The failure of bryozoan larvae to develop in copper solutions capable of accelerating metamorphosis (Lynch, 1949a) should be expected, according to the enzyme hypothesis, for copper would apparently be disastrous not only to the larvae but to the development of adult structures as well.

The hypothesis that autolysis initiates metamorphosis and that this process is accelerated by vital dyes leaves much to be desired. Huxley (1922) could find no evidence for believing that cytolyzing enzymes are a necessary hypothesis for explaining the resorptive dedifferentiation that occurs in hydroids and in *Perophora*. Grave (1935) likewise could find no support for Berrill's theory (1929) that phagocytosis of the tail of ascidian tadpoles is the initiator of metamorphosis. If factors causing the acceleration of bryozoan metamorphosis are listed opposite those bringing about retardation, it seems possible that the "colloidal theory of calcium release" may explain most of the facts. Accelerating agents may conceivably effect metamorphosis by a direct action on protoplasmic viscosity.

Accelerators of metamorphosis:	Inhibitors of metamorphosis:
Heat (Marcus, 1926; Lynch, 1949b)	Cold (Lynch, 1947)
Hypertonic solutions (Lynch, 1949a)	Hypotonic solutions (Lynch, 1947)
Absence of magnesium	Excess of magnesium (Lynch, 1949a)
Moderate amount of diffuse light (Grave, 1930)	Absence of light (Lynch, 1949b)
Copper (Lynch, 1949a)	Excess of potassium (Lynch, 1949a)
Large excess of calcium	Absence of Na, K or Ca in three-ion combinations
Vital dyes	A pH below 6.0 (Lynch, 1949a)

It seems more than merely coincidental that inhibitors of metamorphosis, such as cold sea water, an excess of magnesium or potassium, are also agents that have an anaesthetizing effect. Interpreted in terms of the calcium-release theory, inhibition of metamorphosis may be merely a kind of anaesthesia, reducing the activity of larvae to a minimum and thus forestalling the unfavorable effects of starvation. (For the narcotizing effects of magnesium and potassium see Heilbrunn, 1943, p. 520.) The absence of calcium, at least, should accentuate the anaesthetizing effects of magnesium (Heilbrunn, 1943, p. 531); what effects the absence of sodium or potassium have on protoplasmic viscosity are not well known. It seems logical to attribute the inhibitory effects of hypotonic sea water on the larvae of *B. neritina* to a decreased protoplasmic viscosity caused by absorption of water. (If, however, sea water is diluted by 50% with distilled water and larvae are placed in it for ten minutes and then returned to sea water, metamorphosis and zooid formation take place.) Accelerating agents, on the other hand, rather closely parallel those that stimulate protoplasm or cause a clotting of the interior of cells. Thus, hypertonic solutions, which accelerate metamorphosis, are known to cause calcium release from the cortical layer resulting in a concomitant increase of the viscosity of the inner protoplasm. Since light can also release calcium and increase the viscosity of the interior protoplasm (Alsup, 1942), an absence of light may presumably have the opposite effect—hence the inhibiting effects of darkness. (It is interesting to note that Knight-Jones, 1951, observed that the trochophores of *Spirorbis* behave like the larvae of *Bugula* in darkness; both attach to the surface film predominantly. In fact, the pre-fixational activities of *Spirorbis* are so remarkably similar to those of *Bugula* that the parallel suggests that behavioral patterns may be preserved through evolutionary changes.) The action of vital dyes may be interpreted, perhaps, as direct effect on protoplasmic viscosity rather than an indirect one by activating proteolytic enzymes. The studies of Alsup (1941, 1942) on the photodynamic action of rose bengal and eosin on protoplasmic viscosity of the interior of cells, increasing it in the presence (but not in the absence) of light, suggest that neutral red and methylene blue may have effects similar to the dyes he used. All these dyes seem to affect protoplasm in much the same way. The writer found eosin effective in initiating metamorphosis, but it was even less potent than methylene blue, and Hassett (1941) found that rose bengal, eosin, neutral red and methylene blue all had similar effects on the light responses of *Peranema tricophorum*. Whether vital dyes affect the protoplasm directly or only indirectly via an enzyme system is problematical, for Heilbrunn (1943, p. 538) stated that "the clotting of protoplasm may well be related to some proteolytic enzyme action." At present it is generally believed that dyes with a photodynamic action affect exposed —SH groups of the protein molecule. (*Cf.*, Calcutt, 1951.) The observations of Angerer (1937, 1942) that copper chloride (an accelerator of metamorphosis) increases the viscosity of sea urchin eggs and of the protoplasm of *Amoeba dubia* may indicate that copper also has a direct effect on larvae rather than an indirect one by inactivating larval enzymes. At any rate, the hypothesis of Glaser and Anslow (1949), emphasizing the role of copper in inactivating larval enzymes, seems to be inadequate, since it fails to take into consideration the host of other substances that accelerate metamorphosis.

There is, therefore, some cumulative indirect evidence for the hypothesis that anaesthetic agents inhibit metamorphosis and that factors causing a release of calcium accelerate it; if it is a tenable one, further experiments should show a general effect of all narcotizing agents. (It is interesting to note that Grave

(1935) observed a narcotizing effect of something in the medium when adrenalin was used in experiments on ascidian metamorphosis; attachment was inhibited.) If coagulating agents can induce metamorphosis, thromboplastic-like substances should hasten the process and heparin should inhibit it. Harding (1951) found that injury substance from minced frog muscle initiated cell division parthenogenetically in *Arbacia* eggs. This substance, according to Heilbrunn *et al.* (1936), is apparently a thrombin-like material. Heilbrunn and Wilson (1949, 1950) found that heparin, a bacterial polysaccharide acting like heparin and dicumarol, inhibited cell division in the eggs of *Chaetopterus*. None of these substances has been tried for inhibiting effects on bryozoan larvae, but injury substance made from frog muscle according to the procedure outlined by Harding (1951) not only failed to accelerate metamorphosis but actually inhibited it. Seven or eight trials with this substance, extracted from frog muscle on four or five occasions, all gave negative results. At a pH of 4.5, found to be effective by Harding (1951), the larvae became immobilized immediately and merely disintegrated after a few hours. Since metamorphosis is inhibited at a pH as low as 4.5, injury substance was tried within a pH range of 5.7, 7.6 and 8.3. (KOH and borate buffers were used in raising the pH of the originally acid injury substance) in combinations ranging from 3 cc. injury substance/7 cc. of sea water, the reverse proportions and other mixtures. In all cases, from pH 5.7–8.3 the results were similar. The larvae gradually lost adhesive material from the internal sac, became stuck to one another, eventually disentangled themselves and continued to swim for 8–10 hours. Metamorphosis could not be induced even when neutral red was subsequently added to these media after five hours exposure. Results similar to these were obtained with the whole blood of frogs added to sea water. In some of these experiments with thrombin-like substances the internal sac ruptured completely without being everted; but the process was mechanical and not one in which the larvae actively participated. In all cases the entire medium in which larvae were placed became extremely cloudy because of congealed adhesive material. Yet the fact that these substances did cause emission of attachment fluid might lead to the supposition that clotting agents in exactly the right proportions might prove effective in accelerating metamorphosis. The theory of calcium release would seem to offer some explanation for the accelerating effects on ascidian larvae caused by an extract of muscle tissue of rabbits killed by x-rays (Bertholf and Mast, 1944) and by whole thyroid gland (but not thyroxine), mantle tissue, etc. (Grave, 1935). In fact, Grave stated (1935, p. 288) that, "If muscle tissue of whatever origin should prove to contain an accelerating substance, an explanation might be afforded for the exceptional results of experiment 191, in which an extract of mantle and foot tissue of a snail . . . induced 100% metamorphosis in a group of larvae of *Polyandrocarpa*. . . ." Although Grave considered that the effective agent in muscle might be an endoenzyme or ferment, it seems more likely, in view of the recent experiments of Heilbrunn *et al.* (1946), that a thromboplastic-like substance may be involved. Like carcinogens and like parthenogenetic agents, factors influencing metamorphosis are many and varied, apparently having little or no relation to one another. Only extensive experiments in the future can bring the complicated process of metamorphosis nearer to an acceptable solution.

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SUMMARY

1. In calcium-free van't Hoff's solution and both Na-free and K-free mixtures having the other ions in the same proportion as in sea water at a pH of 7.1–8.0, the larvae of *Bugula flabellata* behaved exactly like those in sea water containing an excess of $MgCl_2$. Phototropic reactions were lost, ciliated tissue was shed as material migrated basally from the pallial furrow and metamorphosis did not occur.

2. In isotonic Mg-free solutions ($pH = 8.0$) metamorphosis was greatly accelerated, fixation occurring within 30 minutes to 3 hours in the majority of larvae; these organisms developed well-formed zooids in 10 hours. (Normally only about 77% of the larvae metamorphose by 12 hours.)

3. Neutral red, methylene blue and, to a lesser extent, eosin accelerated metamorphosis. Neutral red, the most potent of the three, was effective in concentrations of 1 : 1,000,000 parts of sea water; methylene blue caused acceleration in concentrations of 1 : 500,000 parts. At a concentration of 1 : 100,000 parts of neutral red the average duration of the natatory period was about an hour.

4. Metamorphosis failed to occur in Ca-free, Na-free and K-free media to which neutral red had been added in proportions of 1 : 100,000 parts. The same concentration of neutral red in isotonic Mg-free media ($pH = 8.0$) had a greater accelerating effect than when the dye was added to sea water in the same proportions. A reduction of temperature to 4° C. to 12° C. of sea water containing neutral red in parts of 1 : 100,000 antagonized the accelerating effects of neutral red. The staining reactions of neutral red and the problems involved in the study of metamorphosis are also discussed.

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THE FINE STRUCTURE OF THE PERITROPHIC MEMBRANES OF CERTAIN INSECTS

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The structure and formation of the peritrophic membrane (p.m.) of many insects have been studied extensively (Wigglesworth, 1950; Richards, 1951). Although the need for more detailed knowledge of the fine structure of the membrane was apparent, the results of early attempts to use electron microscopy were not encouraging (Richards and Korda, 1948). Later, Huber and Haasser (1950) reported some details of the p.m. of *Dixippus;* they found it to be a (p. 397) "more or less regular network, probably fibrous, with a thin film stretched across the holes of the network." Shortly afterwards, networks with meshes approximately 0.2 μ across and composed of fine fibrils were found electron microscopically by Lagermalm, Philip and Gralén (1950) in the excreta of the clothes moth larvae (*Tineola bisselliella*). These networks originated from the insect, and not from the wool which formed its food; it was not proved that they originated from the peritrophic membrane, although this seemed likely because of the similarity between these networks and those described by Huber and Haasser. Huber (1950) reported further details of the structure of the p.m. in *Periplaneta orientalis, Tenebrio molitor* and *Bombyx mori.* In all except the last the characteristic networks were found.

Cross-sections of the peritrophic membrane examined in the light microscope show it to consist of several loosely adhering layers, each roughly 0.5 μ to 1 μ thick. Since the networks are much thinner than this, it appears that each thicker layer can be further separated into thinner layers.

There are said to be two methods of formation of peritrophic membranes. In one, the membrane originates solely from specialized cells at the anterior end of the midgut and in the other the membrane is said to be produced by delamination of a series of concentric lamellae from the surface of all the cells of the midgut. The first type is said to occur in Diptera and Dermaptera and the second in certain Orthoptera, and in hymenopterous and coleopterous larvae (Wigglesworth, 1950). There is, however, still much uncertainty about the origin of the membranes and the nature of the secretory processes producing them. It is possible that a study of the fine structure will help to clarify this problem and to throw light on the physiology of absorption of food and passage of enzymes.

The present investigation began with the chance discovery in saliva of *Periplaneta* of membranes similar to those described by Huber and Haasser and by Lagermalm, Philip and Gralén. This present paper deals with the fine structure of these membranes and those of other insects, and the problem of the formation of the membranes.

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Experimental

A. Sources of material

Most of the material was obtained from the cockroach *Periplaneta americana* (L.) which proved such a satisfactory source that all the illustrations are derived from this insect. However, it is desirable in the first place to determine whether conclusions drawn from a study of this species have a general application; and secondly, since two types of p.m. corresponding to two methods of formation have been described, to examine and compare membranes of these two alleged types. This survey has not yet been carried very far, but the p.ms. of two other species have been found to contain membranes of the same general construction as those of *Periplaneta;* these are *Locusta migratoria* (L.) and *Galleria mellonella* (L.). In the following species membranes were not discovered: the dermapteran, *Titanolabis colossea* (Dohrn); the larva of the blowfly, *Lucilia cuprina* (Wied.); larva of the cabbage white butterfly, *Pieris rapae* (L.), of the dermestid beetle *Attagenus piceus* (Oliv.), and the worker honeybee, *Apis mellifera* (L.). A negative finding, it is emphasized, must be regarded with reserve since an element of chance enters into a search of this nature, and a more extensive search may lead to a positive result. It is not, therefore, possible to state with certainty yet whether two varieties of membrane exist.

Because networks had been previously found in *Tineola* excreta (Lagermalm, Philip and Gralén, 1950), several examinations were made of aqueous suspensions of this material without finding other than traces of fibrils.

B. Preparation of material for electron microscopy

Two procedures were suitable for the preparation of material from *Periplaneta* for examination in the electron microscope: (1) stimulation of the insect to cause saliva to exude from the mouth; and (2) dissection of the peritrophic membrane followed by its disintegration in water.

1. Saliva. The oral exudate from *Periplaneta* consists of a clear fluid which was dried directly on the ordinary specimen screen. The dried screen was washed several times with water in order to remove soluble salts, etc. This technique gave quite clean fields and about one quarter of all screens so prepared yielded membranes.

Following the discovery of membranes in *Periplaneta* possible sites of origin were considered, although their similarity to the structures described by Huber and Haasser (1950) made the peritrophic membrane seem the most probable. Proteinase activity could not be detected in the saliva as might have been expected if the contents of the midgut, which are certainly regurgitated into the crop (Day and Powning, 1949), had been able to reach the mouth parts. The possibility, therefore, that the chitinous intima of the salivary reservoirs or crop might be the source of the membranes was explored. Membranes were searched for in crop scrapings and in the contents of the salivary reservoirs, without success. However, when p.ms. were dissected and treated according to the method described below, abundant membranes were immediately discovered. These were far more extensive than the fragments found in saliva and more details of their structure were apparent. It seems certain that the networks of the saliva were in fact fragments of the p.m., thus indicating regurgitation of midgut contents and suggesting that the inability to detect proteinase activity in the saliva was due to deficiencies of the technique.

2. Dissection. Dissection of the p.m. proved the most reliable source of material. The membrane and contents were dissected from the midgut and allowed to stand in water at about 1–2° C. from a few hours to several days with occasional shaking. Finally, vigorous shaking dispersed the membrane into fragments. After the coarser fragments had settled, drops of the permanent suspension were dried on the specimen screens.

The material obtained from all of these methods of preparation has been subjected to the action of the digestive enzymes of the insect and this should be kept in mind when considering the micrographs.

C. Electron microscopy

The microscope used was an R.C.A. Type EMU, and standard methods of specimen mounting, shadowing and examination were employed. Most screens were shadowed with uranium before examination to facilitate discovery of the p.m. fragments and all the plates reproduced here illustrate such specimens. However, the general structural features of the membranes were visible without shadowing. Attempted staining with phosphomolybdic acid did not result in any differentiation of structure.

RESULTS

Occasional thick sheets were noted in aqueous suspensions of dissected p.ms. These were too thick for examination with 50 K.V. electrons. It was assumed that such material represented the total p.m., *i.e.*, the layers visible as separate sheets in the light microscope, not resolved into components. At the edges of such sheets signs of fraying into thinner membranes and fibrils were sometimes seen.

In preparations derived from saliva or directly from the dissected membrane, the most commonly occurring and easily recognized constituent is the "*regular fibrillar network*" (Figs. 1 and 2), as this structure will be referred to in this paper. Other apparently different kinds of membranes were also found, although these may really be variants or defective forms of the regular networks. For example, a honeycomb-like formation ("*honeycomb network*") was occasionally observed (Fig. 3), and frequently superimposed upon the regular networks there occurred a distinct layer of irregularly arranged fibrils ("*irregular meshwork*") (Fig. 6). Adhering to most membranes and often interpenetrating the fibrillar components was found an amorphous ground substance which, since it seems to be partly removed by the procedures associated with preparation, may consist of a less resistant substance than the fibrils. The electron micrographs alone do not afford

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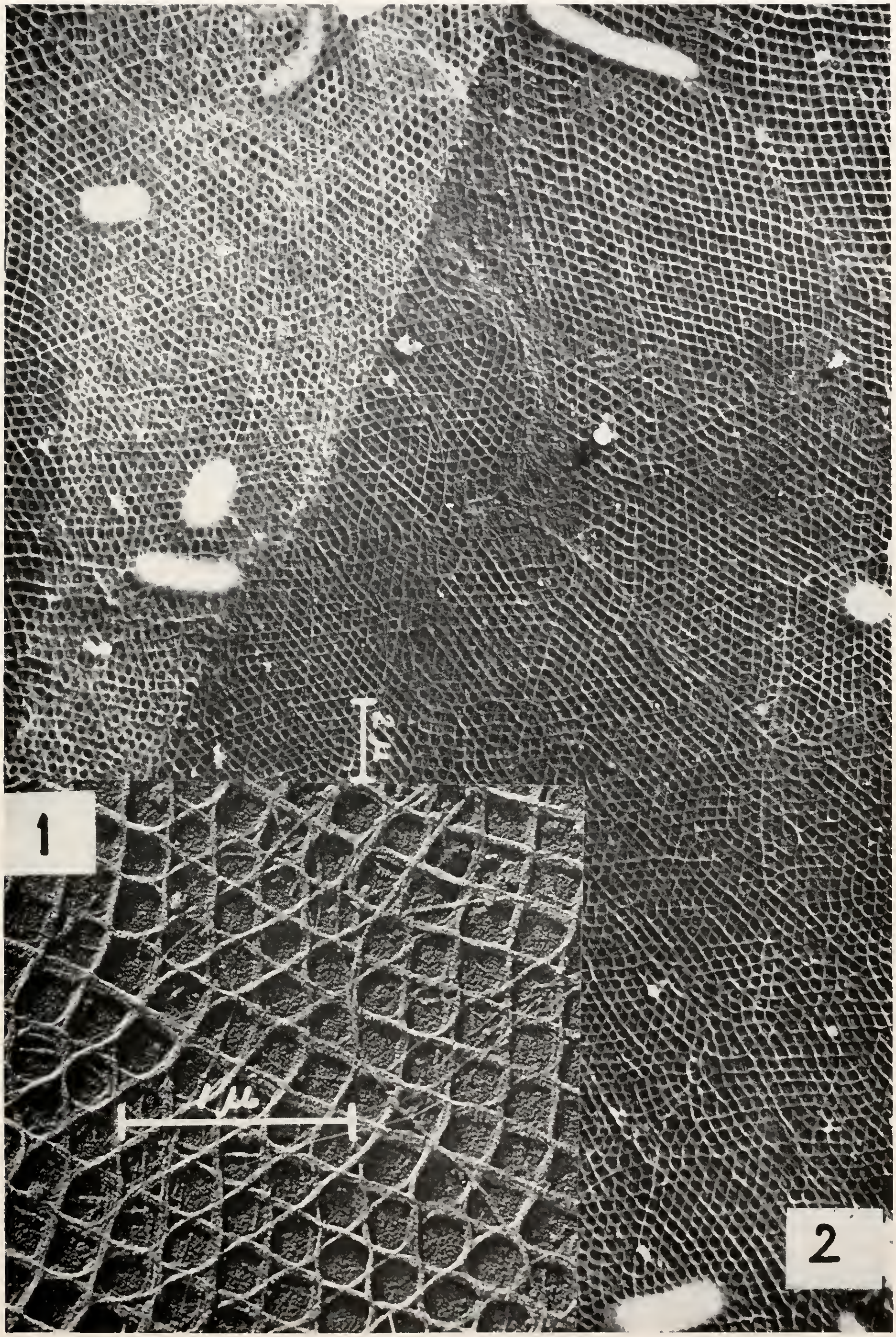
FIGURE 1. Fibrillar membrane from *Periplaneta*. Note areas with approximation to hexagonal symmetry and various types of defective structures. Each strand of the meshwork consists of several fine fibrils. Magnification: 32,000 ×.

FIGURE 2. Extensive area of the fibrillar membrane. Notice folds and structural irregularities. The size of the mesh may be compared directly with the bacteria also present. Magnification: 5200 ×.



Figures 1–2

definite proof that this amorphous substance is a structural constituent rather than a contaminant. However, since it seems to assist in maintaining the coherence of the total formation and is so thoroughly imbedded in the irregular meshwork, it will be assumed that it is a genuine component.

The regular fibrillar networks

The characteristics of these membranes can be appreciated from a study of Figures 1, 2 and 4. They are built from long straight strands or groups of fibrils of diameter probably less than 100 Å and of indefinite length. Ideally the structure tends towards hexagonal symmetry which can be seen in certain portions of the network shown in Figure 1. There are three sets of fibrils placed at 60° and forming triangular or hexagonal meshes (Fig. 8D). Commonly the structure is defective. A set of fibrils may be lacking, resulting in four-sided meshes (Fig. 8E), or the shape may be distorted. The number of separate fibrils composing a strand appears to vary, but is usually about four. Figure 4 illustrates fibrils protruding from the torn edge of a network. The size of the meshes is variable but on the average the parallel strands are about 0.15 μ to 0.2 μ apart. The networks were commonly found stretched flat across the supporting screens with occasional puckers or folds and frequently covered very considerable areas (*e.g.,* greater than 100 × 200 μ). The individual fibrils themselves seem to be structureless, although occasional ambiguous evidence of periodic nodulation was seen after shadowing.

The component fibrils of the strands are neither twisted together to form the strands nor regularly woven into the meshwork. They seem to form a purely random deposition as would result if a collection of straight fibers were sorted out upon an hexagonal array of pegs.

The irregular meshworks

These formations seem to consist of fibrils similar in size to those forming the regular networks, but are less straight. They appear to be bent and felted together and imbedded in the amorphous ground substance which, by interpenetrating adjacent networks, very probably cements the whole laminated structure together. Occasional areas of ground substance lacking fibrils were noted. Some of the fibrils seem to continue directly into those composing the regular networks. This would suggest that the two types of membranes are essentially similar but that the organization is defective in one case.

The honeycomb networks

As can be seen from Figure 3 these networks present a distinctive appearance. In particular they tend to fracture along a straight line of holes, presenting a clean break distinct from that shown by the fibrillar networks (Fig. 4). They may, however, be of the same basic construction as the fibrillar network but consist of fewer

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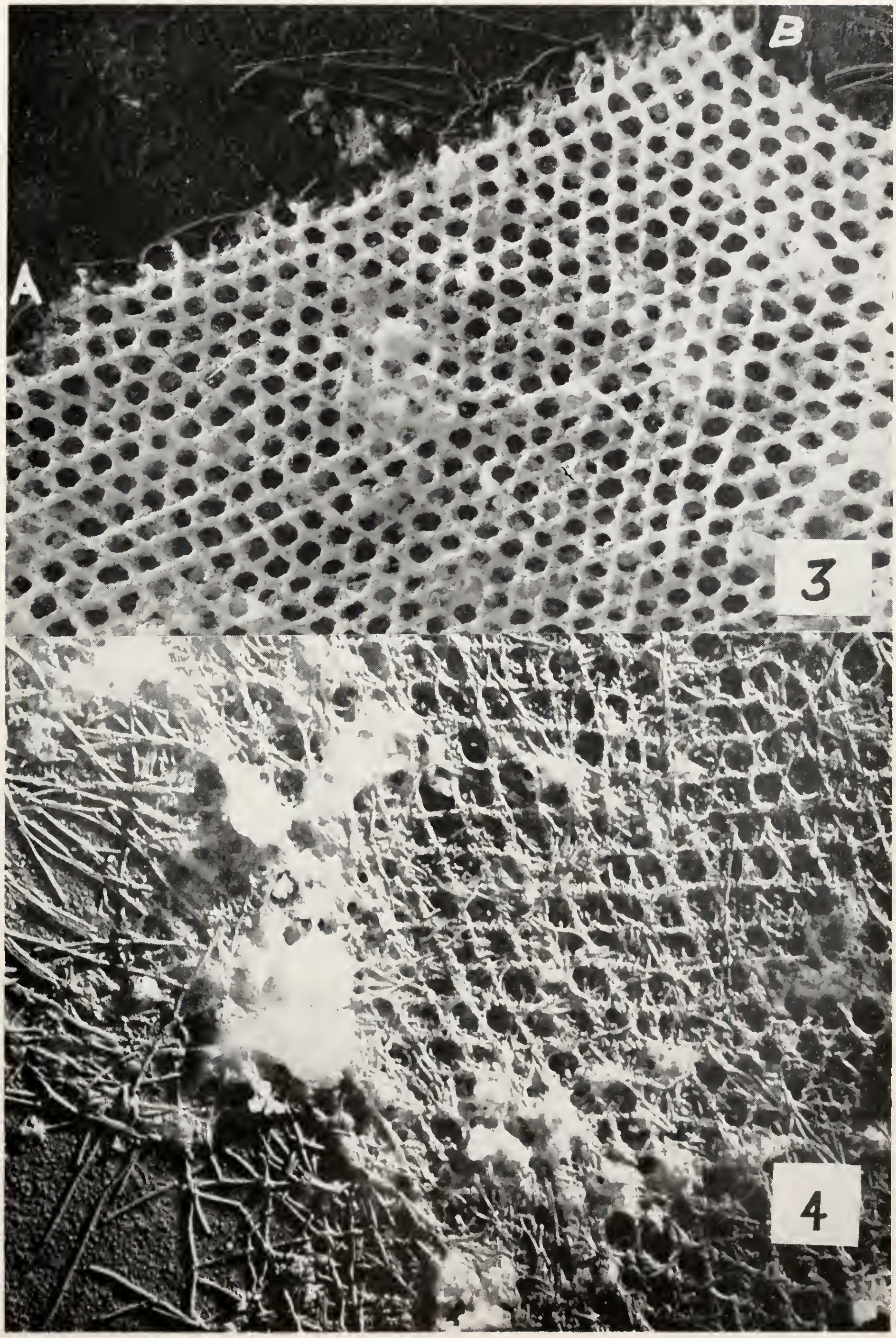
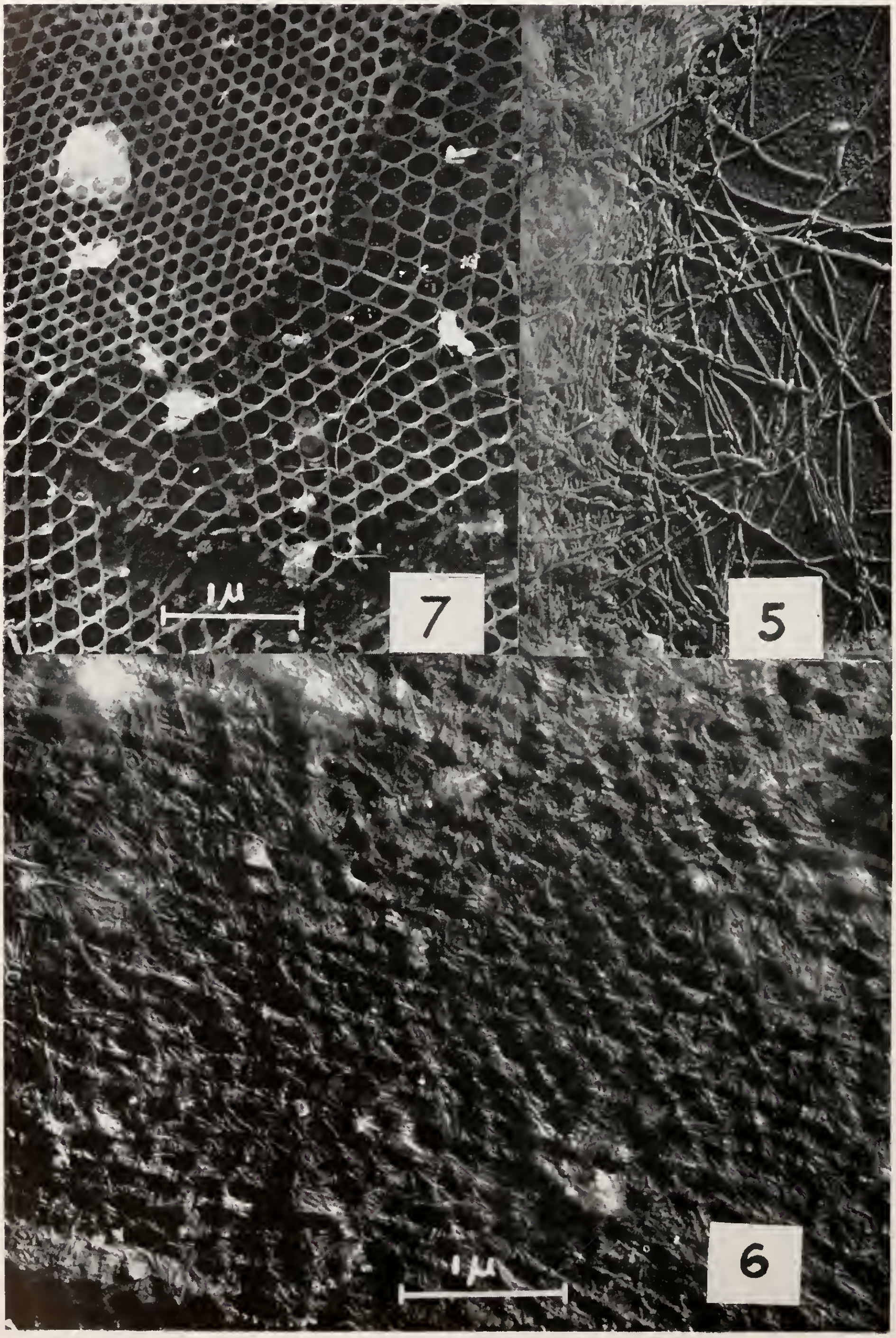
FIGURE 3. Example of "honeycomb network." Note straight edge at AB where membrane has broken. Magnification: 22,000 ×.

FIGURE 4. Edge of fibrillar network showing fraying into fibrils. Patches of ground substance containing fibrils may be seen. Magnification: 22,500 ×.



Figures 3–4



Figures 5, 6, 7

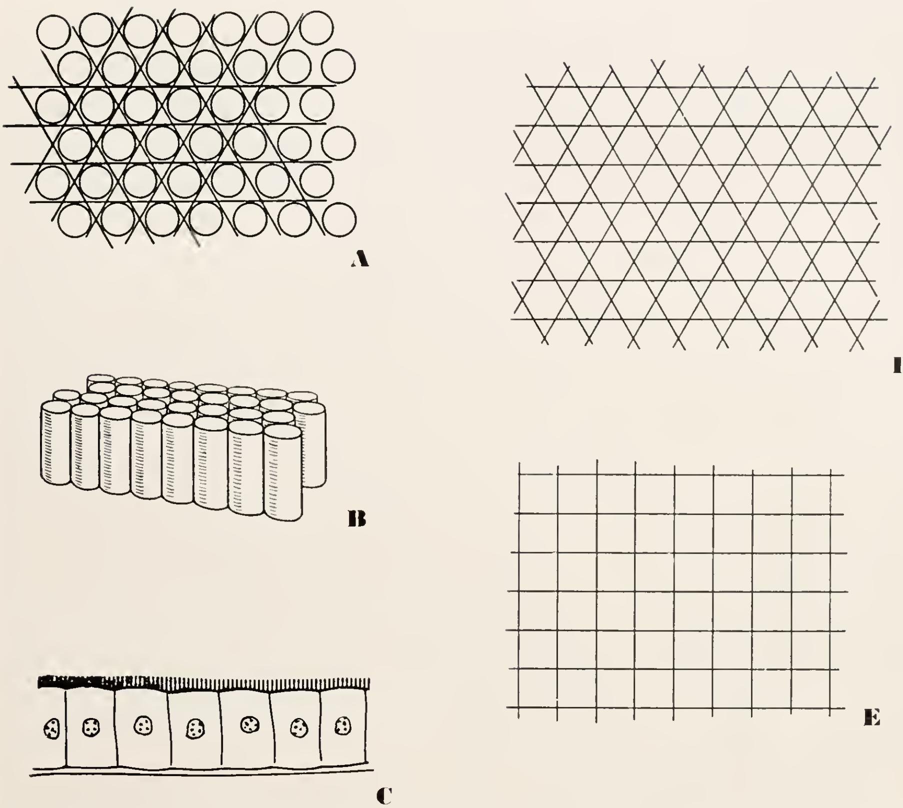


Figure 8. (A) Close-packed arrangement of spherical particles illustrating the formation of the hypothetical template surface having hexagonal symmetry. The way in which fibrils are deposited to form a hexagonal network (D) is indicated. (B) Similar surface of close-packed cylinders. (C) Diagram of striated border of midgut cells which may be formed by the aggregation into coarser fibrils of a finer structure such as shown at (B). (D) Regular fibrillar network as deposited on template of type (A). (E) Square network as deposited on a template of particles in square array formed by distortion of a close-packed array.

fibrils heavily encrusted with the amorphous ground substance. The holes may be either larger or smaller than the average size of the apertures in the regular networks.

Discussion

A. Structure and function of peritrophic membrane

The coaxial lamellae composing the p.m. appear to possess a complex fine structure. Each layer seems to consist of a fine regular network associated with less

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Figure 5. Example of imperfectly developed network. Magnification: 22,500 ×.

Figure 6. Fibrillar network with the irregularly ordered meshwork of fine fibrils partly superimposed. Notice presence of amorphous ground substance. Magnification: 22,500 ×.

Figure 7. A fibrillar network with a honeycomb structure lying above it. Magnification: 19,000 ×.

well defined layers of fibrils and amorphous ground substance. The fibrillar network is obviously that described by other authors (Huber and Haasser, 1950; Huber, 1950; Lagermalm, Philip and Gralén, 1950). The irregular meshwork is always found directly adhering to the fibrillar network, as in Figure 6. Here and there an actual continuity between the fibrils of the irregular mesh and the regular hexagonal network can be traced, suggesting that these two structures are formed from similar materials (fibrils and cement), but that in one case the regular order has failed to develop.

Histochemical studies have shown that both chitin and protein occur in the p.m. Although some doubt may exist as to the origin of the protein, it has been suggested that there is a chitinous framework with protein incorporated (Wigglesworth, 1950; Richards, 1951). This conception of the structure of the total membrane is in good agreement with the details now revealed by electron microscopy. It is most desirable to identify chemically the several components recognized, but unfortunately little in the nature of electron-histochemistry has been attempted up to the present time. In particular, since the average atomic weights of the atoms in chitin and in protein are of a similar order, a differentiation of these two materials on the basis of their electron-scattering power is not possible. Since chitin is usually fibrous, it seems not unlikely that the fibrillar component of the membranes is chitin and that the amorphous matrix or ground substance is protein. This conclusion was also reached by Huber (1950). In accord with the usual role of chitin as a skeletal substance, the fibrillar component may serve a mechanical function as reinforcement providing strength and coherence to the lamellar structure. The ideal arrangement of the fibrils, *i.e.,* three sets of parallel strands placed at 60° to each other, is mechanically adapted to the task of forming a tough membrane not readily torn in any direction.

The protein constituents of the complex, assuming these are represented by the continuous ground substance, might be expected to be the determining factor in the transport of solutes across the membrane. The networks, assuming these are more or less continuous, could of course act as coarser filters and prevent the passage of organisms, which might be able to digest and penetrate the protein component alone. The relative sizes of mesh aperture and bacteria may be seen in Figure 2, although the bacteria apparently entrapped here probably result from contamination occurring subsequent to dissection.

The broad features of membrane structure revealed in the p.m. may possibly prove to be of general occurrence, since the complex, fibrous chitin plus protein, is also found in insect cuticles (Richards, 1951) and closer examination of these may reveal fine fibrils of chitin embedded in an amorphous ground substance of a protein nature. Ribi (1951) has, in fact, reported the occurrence of fine chitinous fibrils in arthropod cuticle.

B. The formation of the peritrophic membrane

Certain aspects of the problem of the formation of the p.m. have been studied in sections by means of the light microscope. This work has been summarized by Wigglesworth (1950). Reference has been made above to the two methods of formation which have been suggested. A consideration of the fine structure of the membrane offers further data relevant to this problem. The features which seem to be of significance are the lamellar structure and the two-dimensional order exhibited by certain of the networks. Both of these features suggest that these particular membranes are examples of exfoliation. In the first place a laminated structure can be readily imagined to form by the shedding of successive sheets from a secreting surface; and secondly, the fine structure of the individual networks could be plausibly explained if we suppose that the fibrils were laid down on a "template" possessing hexagonal symmetry.

It is worthwhile to speculate concerning the essential features of such a template. If the midgut cell surface possesses a pattern having hexagonal symmetry, the simplest method of achieving this is by the close packing of spheres or of circular rodlets. The outer surface could, for instance, be pictured as a close-packed array of spherical corpuscles (Fig. 8A) or of packed rodlets (Fig. 8B). The idea of packed rodlets is attractive because such a formation by aggregating to a coarser structure on fixation would simulate the striated border (diagrammed in Figure 8C) seen to fringe the midgut cells of most insects when viewed in the light microscope. It could then be assumed that the secretion, from which the fibrils separate spontaneously, deposits these fibrils directly in the "grooves" of the patterned surface (Figs. 8A and 8B). Those immediately adjacent to the patterned surface would be formed into the regular network; the excess of fibrils in the secretion more distant from the template surface would be less well ordered and form the mass of irregular fibrils embedded in the amorphous matrix which is found adhering to the regular networks. The matrix in which these fibrils are embedded is probably a second substance present in the secretion which has not the property of forming fibrils. In this way an intimately mixed chitin and protein (?) complex may result. The production of lamellae of structure alternating between a fibrillar texture and an amorphous texture would ultimately depend upon an alternating synthetic activity of the secreting cells which would determine the ratio of the precursors of these components in the secretion.

Since the networks extend over an area greater than that of a single midgut epithelial cell, the hypothetical hexagonal template must preserve the same orientation in adjacent cells. Picken, Pryor and Swann (1947), discussing the similar problem of orientation in cuticles, believe such a coordination unlikely and so are opposed to relating orientation to an underlying organization of the cell surface. However, having regard for the compressed packing of the cells of the columnar epithelium, it seems clear that the hexagonal shape of the cell ends themselves could determine the orientation of the close packing of the particles on their surfaces.

A suggestion similar in principle has been proposed to account for the origin of a two-dimensional array of fibrils observed on the earthworm cuticle by Reed and Rudall (1948) and for the formation of the radiolarian skeleton by Thompson (1942).

The authors are greatly indebted to Mr. J. Farrant for his invaluable suggestions and assistance with the electron microscopy; to Mr. D. F. Waterhouse for providing some of the material; to Mrs. N. Dewsnap for technical assistance; to Mr. L. A. Marshall for the preparation of the text figures; and to many colleagues for helpful discussions.

SUMMARY

1. Some details of the fine structure of the peritrophic membrane of *Periplaneta* and of several other insects have been determined electron microscopically.

2. The membrane appears to be a complex structure of which the most characteristic and resistant component is a fibrillar network. A second component is a layer closely associated with the fibrillar network, consisting of unorganized fibrils embedded in an amorphous ground substance. The layers may be loosely associated and become separated on shaking the dissected membrane in water. The fibrils probably consist of chitin and the ground substance of protein.

3. The fibrillar network generally consists of three systems of parallel fibrillar strands placed at 60° to each other; thus it possesses hexagonal symmetry. A variety of defective arrangements, arising from mesh distortion, suppression of one set of fibrils and other grosser defects, may occur. The diameter of the fine fibrils composing the strands is about 100 Å and there may be several in each strand. The separate strands of a system of fibrils are about 0.15 to 0.2 μ apart. The fibrillar networks are well adapted to the formation of tough sheets, not readily torn.

4. Membranes of this nature were also found in *Locusta migratoria* and *Galleria mellonella.*

5. The nature of these membranes suggests that they are formed by delamination from a surface. This view of the mode of formation may account for the development of the fibrillar arrangement if it is assumed that the fine fibrils are deposited from a secretion onto a surface bearing an hexagonal pattern to act as a template.

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BLEEDING AND COAGULATION IN SOME BERMUDAN CRUSTACEA [1]

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The blood of crustaceans has long been of interest to zoologists and many observations on the coagulation function are to be found in the early literature. Glavand (1948) cites a score of studies prior to 1893, two dozen more between this date and 1920 but only a handful thereafter. These studies consider the nature of crustacean coagulation, contrasting it to that in vertebrates, and a number compare various species in regard to intensity and type of mechanism. Tait (1911) recognized three types of animals: first, those with cellular agglutination alone; second, those with cellular agglutination together with some further clotting of the plasma (*i.e.,* with fibrinogen); and third, those with negligible cellular agglutination but substantial clotting of the plasma. The second group, those with both mechanisms, forms a continuous series of intergrades between the first and third groups with either agglutinating cells or fibrinogen. These distinctions were all based on qualitative observations as to the strength and properties of the coagula since no quantitative measurements of the mass of cellular material or of fibrinogen were made. Even Glavand's recent careful study on the lobster (1948) gave only a rough average value for the normal fibrinogen level in this species. Such information is of interest in evaluating the coagulation function in various invertebrates and in comparing it to that in vertebrates.

MATERIALS AND METHODS

Animals

Some 150 individuals representing 12 species were collected by hand or trapped in an 8-week period in the summer of 1946. The availability of these various crustaceans is indicated in Table I where the numbers, weight range, sex, habitat and mode of capture of these animals are summarized. All specimens were caught personally except for the spiny lobsters which were taken by commercial fishermen and the locust lobsters which came from the Bermuda Aquarium through the kindness of its director, Mr. Louis Mowbray.

A number of other species were collected but not studied because only one or two specimens were available and/or because the species were too small to allow effective collection of blood. These included the ghost crab (*Ocypode arenarius*), the gulfweed crab (*Planes gracilis*), the flat crab (*Percnon planissimum*), the spider crabs (*Mithrax forceps, Microphrys bicornutus* and *Macrocoeloma trispinosum*), petrolisthes (*Petrolisthes armatus*), the red-hermit crab (*Calcinus sulcatus*),

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TABLE I

General characteristics of the species studied

Species	Weight in gm.	No. ♂	No. ♀	With eggs	Location	Capture
Portunidae						
Callinectes ornatus	50–85	9			Sheltered muddy bay, free swimming or buried	Net or trap
C. danae (Bermuda blue crab)	50–140	3	2			
C. sapidus (common blue crab)	165–380	4				
Achelous smithii	100–115				Sheltered channel or open bay	Trap
Piluminidae						
Eupanopeus herbstii	10–59	17	11	3	Sheltered bay; in or on muddy bottom	Trap or under stones
Grapsidae						
Plagusia depressa (cliff crab)	34–130	10	11	4	Rocky open shore, always in water	Net
Grapsus grapsus (rock crab)	25–105	11	14	6	Rocky open shore, in or out of water	Hand or net
Goniopsis cruentatus (Mangrove crab)	18–43	9	8	2	Mangrove swamp, in or out of water	Trap
Gecarcinidae						
Gecarcinus lateralis (common land crab)	1.8–27	19	18	4	Stony island or beach	Under stones
Paguridae						
*Dardannus venosus** (red-veined hermit crab)	56				Sheltered channel	Trap
Palinuridae						
Panulirus argus (spiny lobster)	150–4500				Reef	Trap
Scyllaridae						
Scyllarides aequinoctialis (locust lobster)	720–870				Reef	Trap

* The only large hermit crab listed for this region. In this specimen, however, the right chela was the larger.

the tri-colored hermit crab (*Clibanarius tricolor*), *Glypturus branneri* and the "split thumbs" (*Gonodactylus oerstedii* and *Pseudosquilla ciliata*). (Nomenclature follows Verrill, 1908, 1922.)

The rock crab (*Grapsus grapsus*) was perhaps the most abundant large crustacean on the island but because of its agility and thin shell it was very difficult to capture uninjured specimens by ordinary means. At night, however, these animals could be approached with a light and picked up directly from the rocks where they lay just above the waterline. By this means 5 were taken in 15 minutes on one occasion and 7 in 25 minutes on another, as compared to 5 animals taken alive in two hours by two men during the day. These crabs killed one another in a small container or tank and seemed to survive poorly when this was prevented by removing the chelae. In a large tank with a screened enclosure which permitted them to come out of water, they remained healthy and vigorous for several weeks. Another large grapsoid crab with a heavier shell, the "cliff" crab (*Plagusia depressa*), was taken in considerable numbers although it is listed as "rare" by Verrill (1908). Belying its name, it was never seen above water but as many as 10 to 14 were taken from just below the water line along rocky shores on still sunny mornings.

Bleeding

The animals were bled from the limbs after amputation, usually within 4 to 20 hours of capture. Usually the chelae were cut first and then one of the larger walking legs. The courses of two representative bleedings are shown in Figure 1. Although coagulation occurred quickly in the collecting vial, usually within a minute, it was often many minutes before flow stopped at the limb. Bleeding was encouraged by holding the cut stump downward and by maintaining pressure on the carapace. After the flow had stopped at one limb or following autotomy, another was amputated, usually with a renewed flow, but this procedure was seldom effective beyond the second or third limb (Fig. 1). There was characteristically a quite abrupt end-point beyond which no more blood could be obtained from the animal despite the application of pressure, time or further amputation.

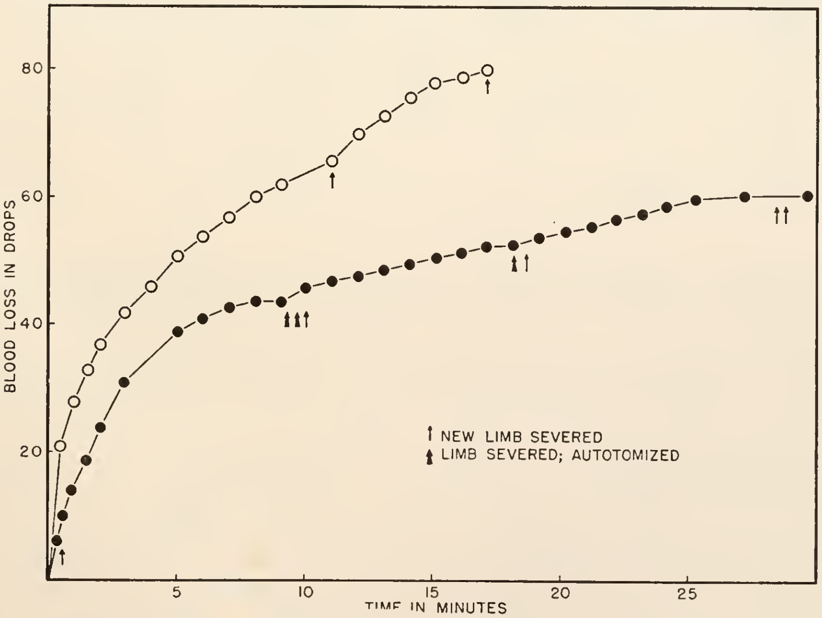

FIGURE 1. Course of bleeding in two representative individuals. Open circles, *Callinectes danae*, 76 g. ♂; bleeding volume 7.0 cc.; average drop, 0.09 cc. Closed circles, *Gecarcinus lateralis*, 27 g. ♂; bleeding volume 3.6 cc.; average drop, 0.06 cc. After an initial rush of blood the bleeding volume was roughly proportional to the square root of the elapsed time ($V = kt^{\frac{1}{2}}$).

Coagulation

The term coagulum will be used to describe the total product of the two processes, the agglutination of cells and the polymerization of fibrinogen into fibrin. In some species this coagulum is largely a cellular agglutinate while in others it is largely a fibrin clot.

Table II

The bleeding volume in various Crustacea

Genus	No.	Ave. wt. g.	Bleeding volume in cc./100 g.		
			Range	Mean	σ
Gecarcinus	35	12	5.6–13.9	9.5	2.8
Grapsus	31	72	4.0–10.8	6.6	1.9
Goniopsis	13	25	4.1–10.5	6.9	2.1
Plagusia	28	75	3.3– 8.8	5.5	1.6
(Grapsidae)	72	64		6.2	(27%)
Eupanopeus	25	29	1.3– 7.1	3.8	1.5
Callinectes	13	130	6.9–18.8	10.5	3.2
Achelous	2	108	6.0– 7.0	6.5	—
(Portunidae)	15	127		10.0	(36%)
(*Brachyura*)	147	53		6.9	
Dardannus	1	56		5.0	—
Panulirus	3*	270	(4.0–10.7)	(6.7)	
	1	4,500		20.0	
Scyllarides	2	800	15.3–18.5	16.9	
Cambarus	7	34	6.0–10.9	8.6	2.0

* Bleeding discontinued before maximum volume obtained.

As the blood dropped from the cut limb it was kept agitated so that the coagulum continuously synerized out as it was formed. In strongly clotting, fibrinogen-containing bloods (*e.g., Panulirus* and *Callinectes*) this was difficult and it was necessary to break up the clot and press out the serum through gauze. Following syneresis the coagulum was washed for half-hour periods successively in 1% sodium chloride and in water to remove soluble organic constituents, largely hemocyanin, and inorganic salts (Morrison, 1947). This step was doubly important in some of these animals because of the high blood salt content and the often imperfect syneresis which resulted in a residue with a considerably higher water content than is obtained from mammalian clots. However, some soluble cellular components were also undoubtedly removed by this extraction process. The synerized and extracted coagulum was then dried at 105° C. for 48 hours and stored in vials between layers of lens paper, since a balance was not available. Later, after a subsequent 48-hour drying period the coagulum was weighed to 0.1 mg. In smaller species the blood from several individuals was pooled in order to obtain a sufficient quantity of coagulum.

Results and Discussion

Bleeding volume

The data on bleeding volumes in the several species are summarized in Table II. Individual values within a species varied over a three-fold range and average values ranged from 3.8 in *Eupanopeus* to 16.9 cc./100 grams of body weight in *Scyllarides.* The values for the Macrurans averaged higher (13 per cent) than the Brachyurans (7 per cent) and of the latter the swimming Portunids averaged somewhat higher (10 per cent) than the others (6.5 per cent). Part of this latter difference undoubtedly derives from the fact that the climbing or crawling crabs bear a considerably heavier weight of shell than do the lighter swimming or walking species. Thus, values for the per cent of body water ranged from 53 and 54 in *Plagusia* and *Eupanopeus* to 76 and 78 in *Callinectes* and *Gecarcinus.* Bleeding volumes expressed as per cent of the body water were, with the exception of *Eupanopeus* (7.0), much more constant, ranging from 10.4 in *Plagusia* to 13.8 in *Callinectes.*

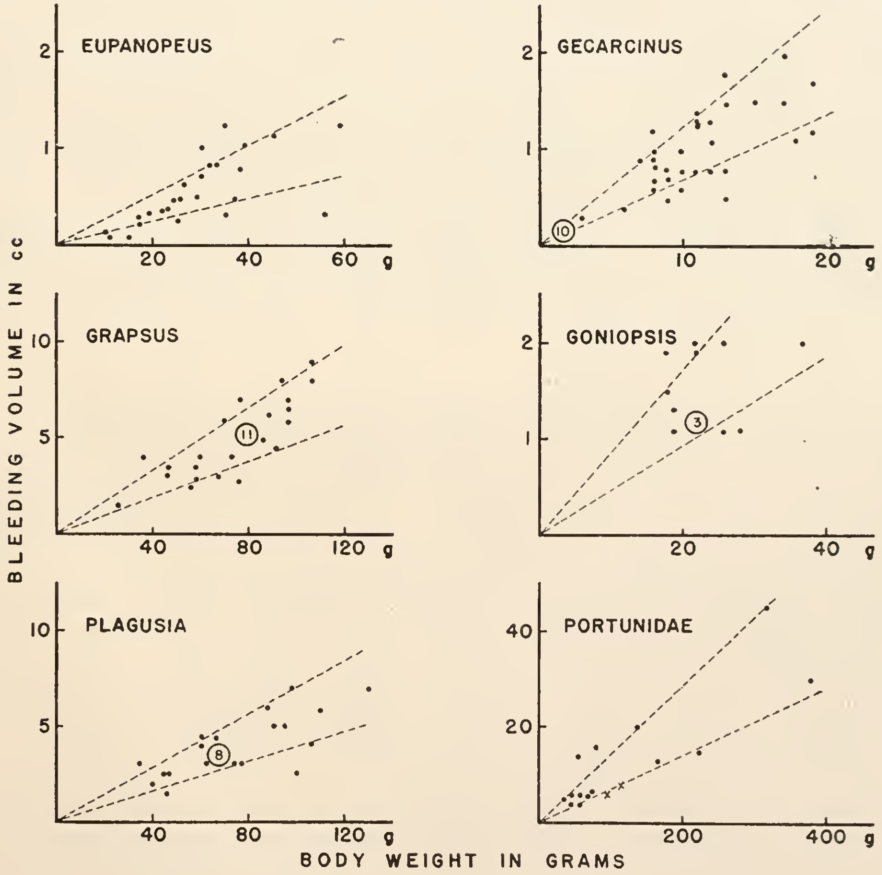

FIGURE 2. Bleeding volume in 6 genera of Crustacea as a function of body weight. Symbols show individual values except the circles which represent pools of the indicated size. Broken lines show standard deviations.

Bleeding volumes for 6 of the species are plotted as a function of body weight in Figure 2. Some tendency toward lower per cent yields in the smaller individuals is seen in *Grapsus* and particularly in *Eupanopeus,* and this may in part account for low bleeding volume in the latter species. A similar group correlation with weight may be seen since the heavier Macrurans have the largest bleeding fraction, the Portunids are next and the smaller Brachyurans are last. This correlation breaks down in comparing the three smallest genera, however, since of these the lightest, *Gecarcinus* (12 g.), has the largest bleeding fraction followed by *Goniopsis* (25 g.) and *Eupanopeus* (29 g.).

These crustacean bleeding fractions of 4 to 17 compare to a total blood volume of 25 cc./100 g. as measured in crayfish (*Cambarus virilis*) by Prosser and Weinstein (1950), using dye dilution methods with T-1824 or thiocyanate. Measurements of the bleeding volume on this same species gave values ranging from 6 to 11 and averaged 8.6 cc./100 g. (Table II). Since this value is only one-third of that obtained by dye dilution, it is evident that much blood is left behind in bleeding. On the other hand, the bleeding volume results from a fairly discrete end point and is reasonably constant. Indeed, the standard deviation of bleeding volumes averaged less than 30 per cent of the mean (20 per cent in *Cambarus*) as compared to 36–38 per cent for the blood volume in the dye dilution study referred to above. It seems possible, therefore, that the bleeding volume may represent some definite fraction of the total blood volume, perhaps that fraction occupying the heart, blood vessels and large sinuses since this would be the most readily expressed. With this concept in mind it is of interest that the bleeding volume bears the same relation to the blood volume (T-1824 or NaCNS) in the crayfish (1:3) with its open circulation as the blood volume (T-1824) bears to the extracellular volume (NaCNS) in the frog (1:3.3) or mammal (1:3–4) with their closed circulation (Prosser and Weinstein, 1950).

Coagulation; general description

The initial step in coagulation, which removed the cellular elements, usually began and was completed within a minute. Before this time the freshly drawn blood was opaque but thereafter it was clear although often strongly colored by hemocyanin and/or other blood pigments. In *Eupanopeus, Gecarcinus* and the Grapsidae this initial coagulum was fairly substantial and little if any further clotting could be distinguished. In *Panulirus,* however, the initial stage was often represented by only a few shreds of material and the first intimation of clotting was a sudden gelation of the solution. If the clotting solution is undisturbed the gel is very transparent, rigid, elastic and synerized only with difficulty. These are all properties which characterize a "fine" clot containing small structural fibers (*ca.* 100 Å) and interstices such as is formed from mammalian fibrinogen under conditions of high pH and fibrinogen concentration (Ferry and Morrison, 1947).

Under physiological conditions mammalian fibrinogen forms a coarser structure than crustacean fibrinogen, a distinction which was first observed by Howell (1916) under the ultramicroscope. Stubel (1920) also described the transformation of invertebrate fibrinogen as into small "granules" rather than into the long "needles" characteristic of mammalian material. In the amphibians and fishes, however, fine structured clots are seen in both gross and microscopic observation (Stubel, 1914). Among the vertebrates this difference in clot structure may be correlated with differences in the solubilities of the respective fibrinogens (Morrison, Scudder and Blatt, 1951). The more soluble fibrinogens found in the lower vertebrates may represent smaller, less asymmetric and/or more highly charged molecules, any or all of which conditions could account for the "finer" clots formed from them. That a similar situation may exist in crustacean fibrinogen is further suggested by the observation of Glavand (1948) that a relatively high gel fraction (*ca.* 1.5 g./1.) was necessary for clotting in these animals. A gel fraction of as little as 0.05 g./1. is sufficient to form a continuous clot in systems with mammalian fibrinogen (Ferry and Morrison, 1947).

Coagulum weights

Average weights for the coagula are summarized in Table III. Values by species ranged from 3.6 g./1. in *Plagusia* to 16.6 g./1. in *Panulirus.* Individual values are very variable, with standard deviations averaging about 50 per cent and with as much as an eight-fold difference being observed in *Callinectes.* Such variation in the coagulating ability of crustacean blood has been noted before. Cuénot (1891) reported that strength of the clot varied much more than the level of hemocyanin in blood from starving crabs and lobsters. Similarly, Glavand (1948) found that the fibrinogen in lobster blood often disappeared entirely during captivity. In this study the two locust lobsters had been held in captivity in the Bermuda Aquarium for a number of months which may account for the smaller coagulum, negligible in one specimen, and the longer clotting time as compared to the spiny lobsters which were freshly trapped. Similar changes in nutritional or other conditions in the natural trapped specimens may be responsible for the high variability observed here. The natural variability of fibrinogen in the lady crab, *Ovalipes,* was apparently so great that Loeb (1903) first reported it to be absent in this species and then subsequently (1904) reported it to be present.

Gruzewska (1932) related lower fibrinogen levels in the green crabs (*Carcinides maenas*) to moulting. Values for the only two soft-shelled (blue) crabs taken in this study were not included in the average values in Table III. These values (2.4 and 2.5 g./1.) are among the lowest found in this group being less than half of the mean value.

The Macrurans formed much heavier coagula (average = 17 g./1.) than the Brachyurans (average = 5 g./1.). This agrees with the qualitative observations of much more effective clotting in European Macrurans as reported by Cuénot (1891) (*Palinurus*), Heim (1892) (*Palinurus, Homarus*), Tait (1911) (*Palinurus, Homarus* and *Astacus*), and Glavand (1948) (*Homarus* and *Nephrops*), and in *Homarus americanus* by Loeb (1904).

Large differences in clotting ability are found among the various Brachyurans. Heim (1892) and Tait (1911) both mention *Portunus puber* as the one species among many crabs which approaches the Macrurans in this regard and Loeb (1904) and George and Nichols (1948) refer to the firm clots in the related *Callinectes.* In Table III it may be seen that the Portunidae do average somewhat higher (5.8 g./1.) than the Grapsidae (4.0 g./1.) but because of the high variability the difference is barely significant ($t = 2.7$). Of more interest, and correlating with the very firm clots often observed in this group, may be the fact that the highest indi-

Table III

Coagulum weights in various Crustacea

Genus	No.*	Coagulum in g./l.			Clotting time** sec.
		Range	Mean		
*Gecarcinus****	10	2.1– 5.6	3.6	1.2	44
Grapsus	17	2.6– 6.2	4.1	1.2	
Goniopsis	5	2.5– 6.3	4.0	2.1	
Plagusia	3	3.0– 4.3	3.6	0.9	35–120
(Grapsidae)	25		4.0	(33%)	
Eupanopeus	7	3.0– 7.2	4.7	1.8	5–90
C. ornatus	8	1.5–13.0	5.0	4.7	60
C. sapidus	3	5.0– 7.4	6.2	1.5	60
C. danae	3	2.5– 9.8	8.6	1.6	60
Achelous	2	2.1– 5.5	3.8	2.8	52–75
(Portunidae)	16		5.8	(43%)	
(*Brachyura*)	63		4.8		
Dardannus	1		15.6		
Panulirus	7	8.6–22.3	16.6	5.0	
Scyllarides	2	(1.8– 8.9)	(5.3)		300
*Cambarus*****	5	1.1– 3.3	2.1	1.0	40–65

* Each value represents a pool of 1 to 6 individuals.

** Blood delivered from limb into a glass vial; time measured to the first appearance of a clot; values represent 1 to 3 observations.

*** Excluding value of 1.5 g./l. for a pool from 10 very young (average weight = 1.8 g.) individuals.

**** Commercially supplied individuals from Wisconsin. Time after capture not known.

vidual values (to 13.0) were found in the Portunidae. Only one Anomuran species was studied. The single *Dardannus,* a large hermit crab, had a higher value (15.6 g./l.) than any of the Brachyurans. This may correlate with the lesser defense against injury provided by its soft body.

Agglutination vs. clotting

In this study measurements of fibrinogen alone were not made but only of the extracted coagulum which also contained cellular components. One might estimate the fibrinogen level in those animals possessing it by subtracting from the weight of the coagulum, an amount equal to that in those species which are considered not to have fibrinogen. But a simple correction of this sort is of questionable validity since it assumes that the various species have comparable amounts of cellular material. This is certainly not the case since Yeager and Tauber (1935) found cell counts ranging from $5 \times 10^3/\text{mm.}^3$ in *Talorchestia* to $54 \times 10^3/\text{mm.}^3$ in *Callinectes* (average $= 18 \times 10^3/\text{mm.}^3$ in 13 species of crustaceans) with an individual standard deviation of 30 to 60 per cent. Similarly, George and Nichols (1948) found a hematocrit value of 1.0 per cent in *Callinectes* but only 0.25 per cent in *Libinia*. The species with distinct fibrinogen clotting also appear to have more cells. Although these additional cells do not necessarily represent agglutinating components they will nonetheless become enmeshed in the clot structure and contribute to the weight of the coagulum. Still, even in *Callinectes,* the form most abundantly supplied with cells, the dry cell weight presumably amounts to only 20–25 per cent of the cell volume or 2–2.5 g./l. and the extracted dry weight should be even less. In *Libinia* and other species with fewer cells, a value of only 1/2 to 1/4 the above or 0.5–1.2 g./l. is expected. Since average values of about 4 g./l. (3.6–4.6 g./l.) or 3 to 8 times the above estimate were found even in the five lowest species in Table III, it would appear that considerable material had been adduced to the cellular components from the plasma even in those species which show only an "initial" coagulation phase and which formerly had been considered to have no fibrinogen in their plasma. However, although this unidentified material precipitates from plasma on the shedding of blood it should perhaps indeed not be considered as fibrinogen since it does not form the gel structure which we are accustomed to associate with fibrin.

Using a correction of 1–2 g./l., the dry cell weight estimated above, we arrive at a mean value of 3 g./l. for the plasma component in *Eupanopeus, Gecarcinus* and the Grapsidae. In the spiny lobster, *Panulirus,* the fibrinogen value is five times this amount or 15 g./l. and individual values range up to 20 g./l. Glavand (1948) reported values for the fibrinogen level in the common lobster, *Homarus vulgarus,* ranging from 2.9 to 10.9 g./l. and stated (p. 96) that "fibrinogen of about 0.4 per cent (4 g./l.) in the citrated plasma is as a rule found." These values are considerably lower than those in the spiny lobster but the Palinuridae, *i.e., Palinurus,* have been previously cited as having the most strongly clotting blood of any of the crustacea, including the other Macrurans (Tait, 1911).

Mammalian vs. crustacean fibrinogen levels

It is of considerable interest to compare the fibrinogen level in mammals to that in these crustaceans. The clot formed by the blood of *Panulirus* is certainly equal and probably superior to an ordinary mammalian clot in strength and rigidity. However, this appears to be achieved only by the use of a considerably larger amount of material (15 g./l.) since the normal fibrinogen level in mammals is at only 2–4 g./l., although in some species, *e.g.,* swine and cattle, values up to 8–10 g./l. have been reported. In the less effective of the crustaceans the coagulum weight was roughly equal to the fibrinogen level in many mammals, but in functional terms of gel formation the two groups cannot even be compared.

In terms of variability a striking contrast is seen between the mammals and the crustaceans. Gram (1921) in measurements on 50 men and women found an average value of 2.8 g./l. with a *range* of ± 30 per cent. Similarly, Ham and Curtis (1938) found an average value of 2.5 g./l. with a *range* of ± 28 per cent in 38 normal men and women. These values of ± 30 per cent for the *range* of variation in sizable groups of men compare with *standard deviations* of about 40 per cent in the Crustacea. The latter value is low since a number of the individual values upon which it was based represented pools of blood from up to six individuals. The difference is even more striking in single individuals which in the crustaceans can lose all coagulation function (Glavand, 1948) but which in man vary by only ± 4–8 per cent from the mean over periods of more than a year (Gram, 1921; Ham and Curtis, 1938). It should be noted, however, that fibrinogen levels of up to 7 g./l. have been recorded in man in response to a variety of infectious diseases (Ham and Curtis, 1938).

Coagulation and hemostasis

Some inverse correlation between autotomy and clotting ability may be seen in the fact that the heaviest clots came from a genus (*Panulirus*) in which it is difficult to induce the autotomy of even a single limb while the lightest clots came from genera (*Gecarcinus, Eupanopeus* and the Grapsidae) which are very prone to autotomy. Tait (1911) considered this proposition, *i.e.,* that these two hemostatic mechanisms might substitute for one another, and although most of his data

TABLE IV

*Hemostatic influences in various arthropods**

Genus	Size	Shell	Autotomy	Cells	Fibrin	Σ+	Reference**
Limulus	+	++	0	++++	0	7	d
Libinia	++	++++	+	+	0	8	d,g
Maia	(++)	+	+++	+	0	7	b,g
Gecarcinus	+++	+	++++	+	0	9	a,g
Goniopsis	+++	++	+++	+	(0)	9	a
Plagusia	++	++++	++	+	(0)	9	a
Grapsus	++	+++	++	+	(+)	9	a
Eupanopeus	+++	++	++	++	0	9	a,g
Cancer	++	+++	+	+	+	8	e,f,g
Carcinides	++	++	++	+	+	8	e,f,g
Callinectes	++	++	++	+	++	9	a,d,g
Portunus	++	++	++	+	+++	10	c,e,g
Dardanus	+++	+	(+)	?	+++	8	a
Astacus	+++	++	+	0	+	7	e,c,f,g
Cambarus	+++	++	+		+	7	a,g
Homarus	+	+++	+	+	++	8	d,f.g
Scyllarides	+	++	++		++	7	a
Panulirus	+	+++	+	(0)	+++	7	a
Palinurus	+	(++)	++	0	+++	8	b,c,e,g
Isopods	+++	+	0	?	+++	(7)	e

* Average adult size as *small* (< 60 g.), +++; *medium* (60–300 g.), ++; or *large* (> 300 g.), +. Shell weight as *light* or *absent*, +; *moderate*, ++; *heavy*, +++; or *very heavy*, ++++. Tendency toward autotomy as *very strong*, ++++; *strong*, +++; *moderate*, ++; *weak*, +; or *absent*, 0. "Cellular agglutinate" (initial coagulum) or fibrin clot as *heavy*, +++; *moderate*, ++; *light*, +; or *negligible*, 0.

** References: a, this study; b, Cuénot (1891); c, Heim (1892); d, Loeb (1903); e, Tait (1911); f, Glavand (1948); g, Wood and Wood (1932).

were affirmative he felt that two notable exceptions invalidated the principles. Possibly he attempted too great a simplification since fibrinogen and autotomy are not the only factors which affect the potential loss of blood in crustaceans. The agglutinating cellular constituents certainly facilitate hemostasis and the strength of the shell must be important in preventing injuries which might cause blood loss. The over-all size of the individual may also be important since a given thickness of shell will afford less protection to a large animal than a small one. Further, bleeding from a large limb would be more diffcult to staunch than from a small limb. Indeed, in the smaller Crustacea it was often not possible to obtain even a drop of blood from the severed appendage.

The interrelations of these several factors in a number of arthropods are summarized in Table IV where arbitrary grades of intensity have been assigned to the several quantities. The sums of these various values are tabulated in column 6 and show reasonable constancy for the various species. Thus, while the spider crab, *Libinia,* has neither fibrinogen nor effective power of autotomy it is very strongly protected by its shell. *Gecarcinus,* on the other hand, which has protection from neither shell nor fibrinogen has a highly developed power of autotomy. Again, the spiny lobster with little capacity for autotomy and not too heavy a shell in relation to its large size possesses a high concentration of fibrinogen while *Limulus,* with similar disadvantages, compensates by a copious cellular agglutination. Other crabs, *Callinectes, Carcinides* and *Grapsus,* in contrast, appear to utilize all of the mechanisms but each in moderate extent. In brief, then, hemostasis appears to be an important function in these animals, but one which may be accomplished either singly or in concert by a variety of very different means.

SUMMARY

1. Maximum bleeding volumes and coagulum weights were determined on more than 150 individuals of 12 species of Bermudan crustaceans.

2. Average values for the bleeding volume ranged from 3.8 cc./100 g. in *Eupanopeus* to 16.9 cc./100 g. in *Scyllarides* and averaged 7.2 cc./100 g., an amount equal to roughly one-third of the true blood volume in crustaceans but about equal to the blood volume in vertebrates.

3. Average values for the weight of the extracted and dried coagulum representing the sum of fibrin and cellular constituents ranged from 3.6 g./l. in *Plagusia* to 18.1 g./l. in *Panulirus* with large individual variation in most species.

4. Even in animals previously considered to possess only an "initial," cellular phase of coagulation, the coagulum weights were 3–8 times greater than the estimate of the maximum cellular contribution and it was concluded that plasma components must be of importance in these species.

5. In species possessing considerable amounts of fibrinogen a "fine-structured" clot is formed resembling that in lower vertebrates rather than that in mammals. The mechanical properties of this clot compare favorably to the mammalian clot but 3–6 times as much fibrinogen is employed.

6. A comparison of species with regard to the various factors modifying hemostasis, *i.e.,* fibrinogen, agglutinating cells, autotomy, size, and shell strength shows a compensation whereby animals lacking one or more mechanisms were particularly strong in others.

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RESPIRATORY METABOLISM OF AMPHIBIAN GASTRULA EXPLANTS

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Chemical embryologists suppose that familiar differences between the *morphological* behavior of the dorsal side of an amphibian gastrula and that of its ventral side are associated with causally significant differences in their *metabolic* behavior. But numerous attempts to confirm this plausible hypothesis have yielded results that are somewhat disappointing. Several items contribute to this undesirable state of affairs: crucial technical difficulties in handling gastrula explants are only now coming to be explicitly recognized; there is a seeming reluctance among chemical embryologists to repeat the work of others, so that probably unwarranted conclusions are currently accepted; and, worst of all, even the most carefully obtained results have not been suggestive of really fruitful explanatory hypotheses.

Nevertheless, there seems to be open no course other than to continue piecemeal the biochemical exploration of gastrulae in the hope that useful information will be obtained. In accordance with this view, we have (a) reinvestigated the confused question whether or not there is a dorso-ventral differential in respiratory rate in amphibian gastrulae, (b) studied the respiratory response of gastrulae tissues to the action of metabolic poisons that suppress gastrulation, in the expectation that dorsal and ventral explants might respond differently, and (c) tried to confirm the interesting report of Boell, Needham and Rogers (1939) that anaerobic carbon dioxide production of dorsal explants is about triple that of ventral ones.

METHODS

Explants

Rana pipiens embryos at Stage 10 (dorsal lip; Shumway, 1940) were dissected in full-strength Holtfreter's solution with the aid of glass needles. Each dissected gastrula yielded four explants (Fig. 1); dorsal-left (D,L), dorsal-right (D,R), ventral-left (V,L) and ventral-right (V,R). A serious attempt was made to obtain explants symmetrically located with respect to the dorso-ventral and animal-vegetal axes. Loose yolk cells were removed before and after the explants were healed.

Gas exchange measurements

Measurements of respiratory rate (QO_2: μl oxygen/mg. dry weight of tissue hour at 22° C.) were made with the help of silicon-coated cylindrical Cartesian divers (Holter, 1943) whose average total volume was about 17 μl. Each diver contained the following items:

Two explants of the same class: (D,L) or (D,R) or (V,L) or (V,R)
Medium for explants (full-strength Holtfreter's, no bicarbonate): 0.75 μl
NaOH seal: 1.25 μl
Oil seal: 0.9 μl
Mouth seal: 2.5 μl

With these divers, and employing as a test system autoxidizing cysteine, oxidation rates varying between 0.01 and 0.05 μl/hour could be estimated with a standard deviation of 0.002 (25 experiments).

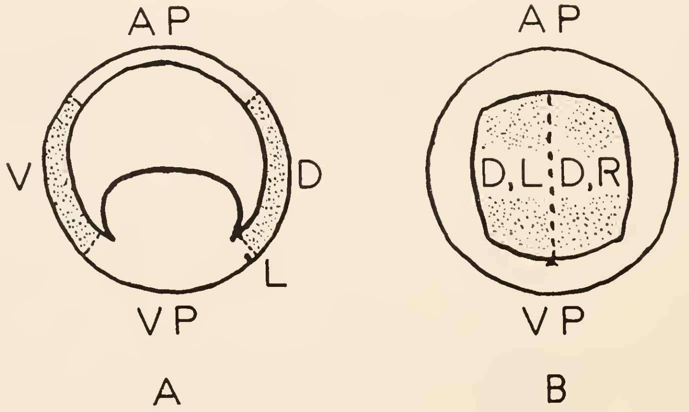

FIGURE 1. A. Diagram of medial sagittal section of a Stage 10 gastrula. Stippled areas show positions from which dorsal (D) and ventral (V) explants were taken. B. Dorsal view of Stage 10 gastrula, showing how dorsal explant is cut into left (D,L) and right (D,R) parts. The ventral explant is similarly treated. Other abbreviations: AP = animal pole; VP = vegetal pole; L = dorsal lip.

Using the same divers, anaerobic carbon dioxide production ($Q_{CO_2}^{an}$: μl carbon dioxide produced/mg. dry weight of tissue/hour at 22° C.) was measured by a method essentially that of Boell, Needham and Rogers (1939). Each diver contained:

Two explants of the same class
Medium for explants (0.03 *M* bicarbonate in full-strength Holtfreter's solution): 0.75 μl
Oil seal: 0.9 μl
Mouth seal: 3.4 μl

Anaerobiosis was insured by the following procedure. After introducing explants into a diver (see below) it was mounted in such a way that it was held upright in a spring clamp with its mouth about two centimeters below the surface of some 95% flotation medium (without taurocholate) contained in a glass cylinder mounted on a vertically-moving rack and pinion (Fig. 2). A stream of gas (95 parts N_2: 5 parts CO_2, previously de-oxygenated over hot copper and washed with water) was then led into the diver with a capillary pipette (as shown) for at least one minute. The diver was then moved along to a second pipette which was used to place the oil seal. The space above the oil seal was next re-gassed for about fifteen seconds, and the diver moved along to a third pipette, which was used to suck out gas from above the oil seal until by test the diver barely stayed afloat in the medium in the cylinder. This latter procedure automatically introduced a mouth seal of the proper volume.[1]

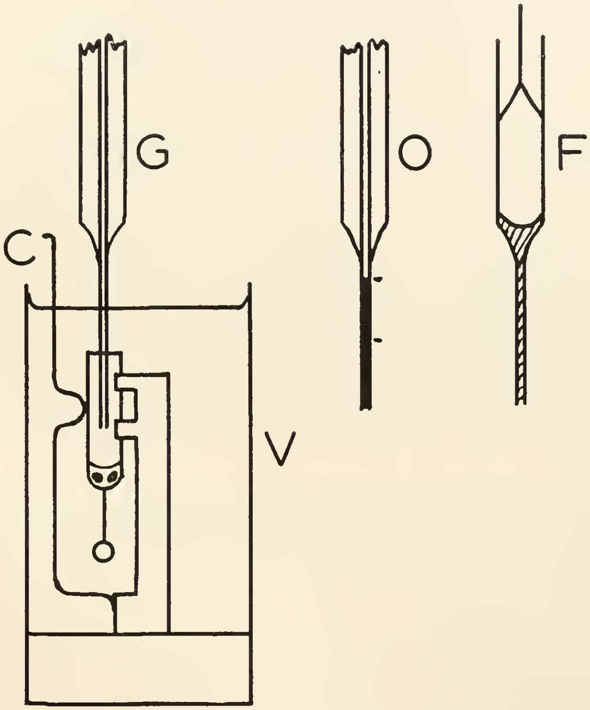

FIGURE 2. Diagram of apparatus used to fill divers anaerobically (not to scale). Diver with two explants shown held by spring clamp (C) in glass vessel (V) filled with flotation medium. Gas pipette (G) in position to gas diver. (O), pipette filled with oil, ready to place oil seal. Two marks represent calibration marks. (F), braking pipette partly filled with flotation medium, ready to insert into diver and remove gas from above oil seal, thus placing mouth seal.

We believe that nearly complete anaerobiosis is thus achieved. For example, a cysteine solution was prepared, samples of which autoxidized under iron catalysis in air-filled divers at the rate of 0.105 μl/hour, whereas control samples in anaerobic divers exhibited a gas uptake of only 0.0009 ± 0.0018 μl/hour (3 experiments). Obviously, oxygen uptake in the anaerobic divers is not significantly different from zero.

When the divers were filled, they were at once transferred to the diver flotation vessels in a constant temperature bath (22° C.) and equilibrated for at least one half hour before readings were begun. Readings were generally made for two hours at intervals of from 10 to 15 minutes.

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[1] This apparatus was kindly lent to us by Dr. L. C. Sze. He used it by filling the cylinder with distilled water kept anaerobic by bubbling the $N_2 : CO_2$ mixture through it. The practice of using flotation medium, in which oxygen is negligibly soluble, is our own innovation.

Loading explants into divers, removing and weighing

Explants were easily and safely loaded into the divers in the following manner. Each diver was filled over-full with the proper explant medium. Explants were then transferred with a braking pipette into the mouth of the diver. When they had sunk to the bottom of the diver, excess medium was removed with a fine capillary pipette.

Following the readings, each diver was removed from its flotation vessel, washed off on the outside with distilled water, and all its seals removed by flushing its neck copiously with distilled water. It was then filled completely with distilled water, and inverted with its mouth under the water contained in a small glass dish until the explants fell slowly out. From the distilled water dish the explants were transferred with a braking pipette, along with about 5 μl water, to small pre-weighed bits of cigaret paper. After drying 30 minutes to overnight at 100° C., the papers and explants were re-weighed to 1 μg on a quartz helix microbalance. On the average, two explants weighed approximately 100 μg.

None of these procedures entailed any danger that the explants would be injured or destroyed by rough handling or by contact with an air-water interface.

Inhibitor solutions

Inhibitor solutions (made up in full-strength Holtfreter's without bicarbonate) employed in the experiments following had different pH values:

Sodium azide: 6.3–6.4
Potassium cyanide: 9.8
Sodium malonate: 7.1–7.3
p-chloromercuribenzoate: 7.9–9.8
Sodium fluoride: 5.8–5.9
2,4 dinitrophenol: 5.0–5.1

Control explants were run in Holtfreter's adjusted with NaOH or HCl to the pH of the corresponding inhibitor solutions. A survey of the results will show that respiratory activity of explants was the same in all these control solutions as in plain Holtfreter's. Furthermore, whole gastrulae develop normally in all of them.

Throughout the inhibitor studies reported in this paper, for each experimental (control) explant used, there was a corresponding control (experimental) explant *from the same gastrula.*

To maintain cyanide concentrations around the explants in the divers (pH 9.8) it was necessary to add cyanide to the NaOH seal as follows:

KCN around explants	*KCN in NaOH seal*
0.0001 *M*	0.09 *M*
0.0005 *M*	0.44 *M*

(*cf.* Umbreit, Burris and Stauffer, 1945; p. 45 ff.).

RESULTS

Respiration in full-strength Holtfreter's solution

In Holtfreter's solution, which for the purpose of these investigations we shall consider to constitute a relatively normal environment for explants, lateral halves of dorsal explants respire at about the same rate ($P > 0.05$):[2]

(1) $QO_2\ (D,L) = 0.30 \pm 0.08$ (20)[3]

(2) $QO_2\ (D,R) = 0.29 \pm 0.05$ (14)

A similar result has been obtained for lateral halves of ventral explants ($P > 0.05$):

(3) $QO_2\ (V,L) = 0.25 \pm 0.05$ (19)

(4) $QO_2\ (V,R) = 0.25 \pm 0.08$ (14)

A further result of these measurements is that we have confirmed the reports of those investigators who have found a dorso-ventral gradient in respiration with respect to dry weight, for dorsal explants respire at a significantly higher rate than ventral explants[4] ($P < 0.001$):

$$(5)\quad \frac{QO_2\ (D,L \text{ and } R)}{QO_2\ (V,L \text{ and } R)} = 1.2 \pm 0.2 \qquad (31)$$

It is not likely, however, that this finding will provide much basis for speculation about the relations of respiratory rate to gastrulation, for it seems that when respiratory rates are expressed in terms of some non-yolk reference component, the respiratory rate of dorsal explants comes out to be identical with that of ventral ones. We do not discuss this question in detail. A forthcoming paper by Sze carefully analyzes the whole problem of respiratory gradients in amphibian gastrulae, and his results shed much light on current disagreements. Our findings are quite in line with his. It thus appears that earlier expectations of discovering embryologically significant differences in the respiratory rates of various gastrula-parts under normal conditions are coming to be less and less warranted by new results. Nevertheless there remains the possibility that differential respiratory responses to less physiological environments may yet be obtained. To this question we not turn.

Respiration in sodium azide (NaN_3)

Sodium azide is thought to act upon biological systems by inactivating the cytochrome oxidase system as cyanide does, or by interfering with the action of some enzyme systems involved in anaerobic phosphate transfer. Azide is a powerful inhibitor of gastrulation in amphibian embryos: both Barnes (1944) and Spiegelman and Moog (1945) have reported the failure of *Rana pipiens* embryos to gastrulate in weak concentrations of azide; and our results confirm theirs, for gastrulae placed at Stage 10 in 0.0005–0.001M sodium azide do not develop past Stage 10^+. Barnes showed also that the respiratory rate of whole gastrulae is sharply reduced (80–90%) in 0.01 M azide. But the respiration of gastrula tissues is sensitive even to weaker concentrations of azide.

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[2] For calculating P-values, Student's t-test has been used throughout.

[3] Parenthesized numerals to the extreme right of equations (1)—(65) indicate the number of experiments performed.

[4] In considering quotients such as (5) it may be of help to know that throughout this paper they are calculated only from the results of experiments in which for each dorsal (ventral) explant used there was a corresponding ventral (dorsal) explant *from the same gastrula.*

In 0.0005 M azide, for example, dorsal explants respire at a significantly lower rate than their controls ($P < 0.01$):

$$(6)\quad QO_2\ (D,R)\ 0.0005\ M\ NaN_3 = 0.08 \pm 0 \qquad (3)$$

$$(7)\quad QO_2\ (D,L)\ \text{control} = 0.23 \pm 0.08 \qquad (3)$$

There is a similar reduction of the respiration of ventral explants ($P < 0.001$):

$$(8)\quad QO_2\ (V,R)\ 0.0005\ M\ NaN_3 = 0.06 \pm 0.02 \qquad (3)$$

$$(9)\quad QO_2\ (V,L)\ \text{control} = 0.20 \pm 0.05 \qquad (3)$$

But there is no indication that the relative rates of respiration of dorsal and ventral tissues are changed by this concentration of azide ($P > 0.05$):

$$(10)\quad \frac{QO_2\ (D,R)\ NaN_3}{QO_2\ (V,R)\ NaN_3} = 1.4 \pm 0.2 \qquad (3)$$

$$(11)\quad \frac{QO_2\ (D,L)\ \text{control}}{QO_2\ (V,L)\ \text{control}} = 1.1 \pm 0.1 \qquad (3)$$

Analogous results have been obtained with 0.001 M azide. The respiration of dorsal explants is greatly reduced ($P < 0.001$):

$$(12)\quad QO_2\ (D,R)\ 0.001\ M\ NaN_3 = 0.09 \pm 0.02 \qquad (3)$$

$$(13)\quad QO_2\ (D,L)\ \text{control} = 0.32 \pm 0.04 \qquad (3)$$

and so is that of ventral explants ($P < 0.001$):

$$(14)\quad QO_2\ (V,R)\ 0.001\ M\ NaN_3 = 0.10 \pm 0.02 \qquad (3)$$

$$(15)\quad QO_2\ (V,L)\ \text{control} = 0.29 \pm 0.04 \qquad (3)$$

But, although statistical analysis indicates a probably significant alteration in the relative rates of respiration of dorsal and ventral explants ($P < 0.05$):

$$(16)\quad \frac{QO_2\ (D,R)\ NaN_3}{QO_2\ (V,R)\ NaN_3} = 0.87 \pm 0.22 \qquad (3)$$

$$(17)\quad \frac{QO_2\ (D,L)\ \text{control}}{QO_2\ (V,L)\ \text{control}} = 1.1 \pm 0.2 \qquad (3)$$

in view of the results obtained with 0.0005 M azide ((6)–(11)) we do not consider this established.

To sum up these results with azide, it appears that if gastrulation of *Rana pipiens* embryos is blocked by a specific action of azide, then either this action is not localized in dorsal tissues or else it is one that respiratory measurements do not reveal.

Respiration in potassium cyanide (KCN)

Cyanide, which inhibits respiration in biological systems by blocking cytochrome oxidase electron transfer, is also a strong suppressor of morphogenetic movements in amphibian embryos. Barnes (1944), for example, found that early gastrulae placed in 0.001–0.002 *M* KCN developed only into mid-gastrulae; 0.0001 *M* KCN only slowed gastrulation. Spiegelman and Moog (1945) partially confirmed Barnes's results: in 0.001 *M* KCN, embryos immersed at Stage 10 developed into late gastrulae (Stage 12); while in 0.005 *M* KCN, embryos immersed at Stage 10 remained at that stage. Our experiments indicate variable results with 0.0005 *M* KCN ranging all the way from complete inhibition of invagination to slight slowing only; while with 0.005 *M* KCN, early gastrulae never get past Stage 11.

Barnes also made some measurements of the effect of KCN on the oxygen uptake of whole embryos. She found a maximal effect at concentrations of 0.001 *M,* at which respiration of gastrulae was inhibited about 98%, with graded lesser effects at lower concentrations.

Our results are partly confirmatory of hers. In 0.0001 *M* KCN the respiration of dorsal explants is reduced by a probably significant amount ($P < 0.05$):

$$\text{(18)}\quad QO_2\ (D,R)\ 0.0001\ M\ \mathrm{KCN} = 0.20 \pm 0.02 \qquad (2)$$

$$\text{(19)}\quad QO_2\ (D,L)\ \text{control} = 0.31 \pm 0.05 \qquad (2)$$

There is a similar effect on ventral explants ($P = 0.01$):

$$\text{(20)}\quad QO_2\ (V,R)\ 0.0001\ M\ \mathrm{KCN} = 0.15 \pm 0 \qquad (2)$$

$$\text{(21)}\quad QO_2\ (V,L)\ \text{control} = 0.27 \pm 0.04 \qquad (2)$$

Statistically speaking, the effect is probably differential ($P < 0.05$):

$$\text{(22)}\quad \frac{QO_2\ (D,R)\ \mathrm{KCN}}{QO_2\ (V,R)\ \mathrm{KCN}} = 1.4 \pm 0.1 \qquad (2)$$

$$\text{(23)}\quad \frac{QO_2\ (D,L)\ \text{control}}{QO_2\ (V,L)\ \text{control}} = 1.1 \pm 0.1 \qquad (2)$$

But in view of the variable results on whole embryos with this extremely weak solution, and in view of the fact that stronger solutions give no such effect, we do not regard this as established by our data. In somewhat stronger (0.0005 *M*) solutions of cyanide, the respiration of explants is reduced a little more for both dorsal (P barely over 0.01) and ventral ($P < 0.01$) explants:

$$\text{(24)}\quad QO_2\ (D,R)\ 0.0005\ M\ \mathrm{KCN} = 0.10 \pm 0.02 \qquad (2)$$

$$\text{(25)}\quad QO_2\ (D,L)\ \text{control} = 0.26 \pm 0.04 \qquad (2)$$

$$\text{(26)}\quad QO_2\ (V,R)\ 0.0005\ M\ \mathrm{KCN} = 0.09 \pm 0.01 \qquad (2)$$

$$\text{(27)}\quad QO_2\ (V,L)\ \text{control} = 0.18 \pm 0.02 \qquad (2)$$

But this effect is not a differential one $(P > 0.05)$:

$$(28) \quad \frac{QO_2\ (D,R)\ KCN}{QO_2\ (V,R)\ KCN} = 1.1 \pm 0.1 \qquad (2)$$

$$(29) \quad \frac{QO_2\ (D,L)\ \text{control}}{QO_2\ (V,L)\ \text{control}} = 1.4 \pm 0.1 \qquad (2)$$

Summarizing: Although it is true that cyanide depresses the respiration of gastrula explants (as some function of its concentration), there is little indication from the respiratory effects that its action in blocking gastrulation is an anatomically localized one.

Respiration in sodium malonate (mal)

Another inhibitor of cellular oxidations is malonic acid which inhibits the conversion by succinic acid dehydrogenase of succinic acid to fumaric acid in the Krebs cycle. So far as we are aware, the only report of the effect of malonate on amphibian gastrula tissue is a report by Brachet and Rapkine (1939) to the effect that dorsal fragments develop normally in 0.025 M malonate. Precisely what this means is not clear.

But in new experiments, we have found that whole embryos placed at Stage 10 in 0.04 M sodium malonate do not develop past Stage 11^+. Furthermore, the respiratory activities of both dorsal and ventral explants are curtailed significantly $(P < 0.01$ in both cases) by malonate in this concentration:

$$(30) \quad QO_2\ (D,R)\ 0.04\ M\ \text{mal} = 0.13 \pm 0.01 \qquad (2)$$

$$(31) \quad QO_2\ (D,L)\ \text{control} = 0.32 \pm 0.03 \qquad (2)$$

$$(32) \quad QO_2\ (V,R)\ 0.04\ M\ \text{mal} = 0.11 \pm 0.01 \qquad (2)$$

$$(33) \quad QO_2\ (V,L)\ \text{control} = 0.26 \pm 0.03 \qquad (2)$$

But this curtailment is not a differential one $(P > 0.05)$:

$$(34) \quad \frac{QO_2\ (D,R)\ \text{mal}}{QO_2\ (V,R)\ \text{mal}} = 1.2 \pm 0.1 \qquad (2)$$

$$(35) \quad \frac{QO_2\ (D,L)\ \text{control}}{QO_2\ (V,L)\ \text{control}} = 1.2 \pm 0.03 \qquad (2)$$

In summary: like other compounds which block dehydrogenase activities (cyanide, azide), malonate reduces the rate of oxygen utilization of gastrula tissues; but, again, the pattern of inhibition furnishes no clue (beyond some very general ones) to the manner in which malonate blocks gastrulation movements. We turn now to a different class of inhibitors.

Respiration in p-chloromercuribenzoic acid (HgB)

Barron and Singer (1945) and Singer and Barron (1945) have shown that p-chloromercuribenzoate is a powerful inhibitor of a large number of enzymes involved in carbohydrate, protein, and fat metabolism. This reagent is thought to act by combining with all available —SH groups in native protein, unlike, for example, ferricyanide which acts only on those —SH groups close enough to form disulfides, or iodoacetate, which in low concentrations combines incompletely with even free —SH groups in native protein.

It is interesting that although 0.0001 *M* p-chloromercuribenzoate is able to prevent further development of gastrulae immersed in it at Stage 10, it has no respiratory effect whatsoever at this concentration ($P > 0.05$ in all cases):

$$QO_2\ (D,R)\ 0.0001\ M\ \text{HgB} = 0.31 \pm 0.06 \qquad (2) \tag{36}$$

$$QO_2\ (D,L)\ \text{control} = 0.36 \pm 0.08 \qquad (2) \tag{37}$$

$$QO_2\ (V,R)\ 0.0001\ M\ \text{HgB} = 0.26 \pm 0.09 \qquad (2) \tag{38}$$

$$QO_2\ (V,L)\ \text{control} = 0.26 \pm 0.08 \qquad (2) \tag{39}$$

$$\frac{QO_2\ (D,R)\ \text{HgB}}{QO_2\ (V,R)\ \text{HgB}} = 1.3 \pm 0.2 \qquad (2) \tag{40}$$

$$\frac{QO_2\ (D,L)\ \text{control}}{QO_2\ (V,L)\ \text{control}} = 1.4 \pm 0.1 \qquad (2) \tag{41}$$

Gastrulae placed at Stage 10 in 0.001 *M* p-chloromercuribenzoate do not develop further. But at *this* concentration the respiration of dorsal explants is sharply and significantly reduced ($P < 0.05$), and so is that of ventral explants ($P < 0.01$):

$$QO_2\ (D,R)\ 0.001\ M\ \text{HgB} = 0.10 \pm 0.02 \qquad (2) \tag{42}$$

$$QO_2\ (D,L)\ \text{control} = 0.28 \pm 0.05 \qquad (2) \tag{43}$$

$$QO_2\ (V,R)\ 0.001\ M\ \text{HgB} = 0.08 \pm 0.01 \qquad (2) \tag{44}$$

$$QO_2\ (V,L)\ \text{control} = 0.23 \pm 0 \qquad (2) \tag{45}$$

However, there is no differential effect ($P > 0.05$) of this —SH inhibitor on the respiration of dorsal and ventral explants:

$$\frac{QO_2\ (D,R)\ \text{HgB}}{QO_2\ (V,R)\ \text{HgB}} = 1.2 \pm 0.1 \qquad (2) \tag{46}$$

$$\frac{QO_2\ (D,L)\ \text{control}}{QO_2\ (V,L)\ \text{control}} = 1.2 \pm 0.2 \qquad (2) \tag{47}$$

Summarizing: p-chloromercuribenzoate will reduce the respiratory exchange of gastrula explants, but the effect upon dorsal and ventral explants is similar. Furthermore, it will block gastrulation in whole embryos. But the effect upon respiration and that upon gastrulation are not associated in any simple manner, for gastrulation may be completely blocked in the total absence of any effect upon the respiration.

Respiration in sodium fluoride (NaF)

Sodium fluoride is known to inhibit the conversion of 2-phosphoglyceric acid into phospho-enol-pyruvic acid by enolase (an enzyme requiring Mg ions) presumably by forming a complex magnesium fluorophosphate in the presence of inorganic phosphate (Baldwin, 1948)—thus preventing the anaerobic production of energy-rich phosphate bonds. In proper concentrations, it is well known to inhibit developmental processes. For example, developing amphibian embryos have been found to be checked by 0.02 *M* NaF but not by 0.005 *M* (Pomerat and Haringa, 1939; *R. pipiens*), and not by 0.01 *M* (Brachet, 1950; *R. fusca*). We have found that in 0.02 *M* NaF gastrulae immersed at Stage 10 do not develop past Stage 14; while in 0.04 *M* NaF they do not develop past Stage 11^+. We have not investigated the effects of weaker concentrations.

But we have not confirmed Brachet's statement (1950, p. 327) that the respiration of amphibian embryos (*R. fusca*) is not fluoride-sensitive; for 0.02 *M* NaF significantly reduces the respiration of both dorsal ($P < 0.01$) and ventral ($P < 0.001$) explants:

$$\text{(48)} \quad QO_2 \text{ (D,R) } 0.02\ M \text{ NaF} = 0.19 \pm 0.08 \quad (3)$$

$$\text{(49)} \quad QO_2 \text{ (D,L) control} = 0.32 \pm 0.04 \quad (3)$$

$$\text{(50)} \quad QO_2 \text{ (V,R) } 0.02\ M \text{ NaF} = 0.14 \pm 0.02 \quad (3)$$

$$\text{(51)} \quad QO_2 \text{ (V,L) control} = 0.24 \pm 0.03 \quad (3).$$

As a result of this treatment, however, there was no significant change in the relative respiratory rates of dorsal and ventral explants ($P > 0.05$):

$$\text{(52)} \quad \frac{QO_2 \text{ (D,R) NaF}}{QO_2 \text{ (V,R) NaF}} = 1.3 \pm 0.4 \quad (3)$$

$$\text{(53)} \quad \frac{QO_2 \text{ (D,L) control}}{QO_2 \text{ (V,L) control}} = 1.4 \pm 0.1 \quad (3)$$

Summarizing: The pattern of fluoride inhibition of respiration of gastrula explants is much like that of other types of metabolic poisons: dorsal and ventral explants are similarly affected.

Respiration in 2,4 dinitrophenol (DNP)

We cannot confirm Andreassi's (1942) report (not available to us; see Brachet, 1950, p. 321) that dinitrophenol accelerates amphibian development; rather, our results support Dawson's (1938) results. Whole gastrulae placed at Stage 10 in 9.5×10^{-6} *M* 2,4 dinitrophenol (α-dinitrophenol; hereafter, DNP) do not develop past Stage 11. In spite of this, however, the tissues of such gastrulae respire at more than twice their normal rate. The respiration of dorsal explants is significantly increased ($P < 0.001$):

$$\text{(54)} \quad QO_2 \text{ (D,R) } 9.5 \times 10^{-6}\ M \text{ DNP} = 0.66 \pm 0.21 \quad (4)$$

$$\text{(55)} \quad QO_2 \text{ (D,L) control} = 0.31 \pm 0.09 \quad (4)$$

There is a similar significant increase in the respiration of ventral explants in DNP $(P < 0.001)$:

(56) QO_2 (V,R) 9.5×10^{-6} *M* DNP $= 0.53 \pm 0.06$ (4)

(57) QO_2 (V,L) control $= 0.24 \pm 0.08$ (4)

But the relative rates of dorsal and ventral explants are not significantly different $(P > 0.05)$:

(58) $$\frac{QO_2\ (D,R)\ DNP}{QO_2\ (V,R)\ DNP} = 1.2 \pm 0.2$$ (4)

(59) $$\frac{QO_2\ (D,L)\ control}{QO_2\ (V,L)\ control} = 1.3 \pm 0.1$$ (4)

In summary: this particular dinitrophenol behaves like other inhibitors of development in failing to act differentially upon the respiratory metabolism of dorsal and ventral explants. Its effect also provides another instance of the ease with which respiratory activity may be dissociated from morphogenetic activity. We now abandon the attempt to affect oxidative metabolism of dorsal and ventral explants differentially and turn to the question whether or not they differ in their anaerobic metabolism.

Anaerobic carbon dioxide production

Boell, Needham and Rogers (1939) reported that under anaerobiosis the CO_2-production of dorsal pieces of amphibian gastrulae is about three times greater than that of ventral pieces. Unfortunately, they believe also that there was an exactly analogous differential in anaerobic ammonia production, and their final values for CO_2-output were arrived at by taking into account the possibility that some 70% of the CO_2 produced was masked by ammonia, which combines with lactic acid produced anaerobically and hence prevents this acid from liberating CO_2 from the bicarbonate medium in which the explants were suspended. But we have recently (Gregg and Ornstein, 1952) tried to cast serious doubt on the reliability of their results: for in early gastrulae we were able to find no significant amount of ammonia produced by gastrula tissues—aerobically or anaerobically—and we have accounted for their ammonia values as artifacts. Consequently, we have felt it necessary to re-investigate the question of anaerobic CO_2-production by gastrula explants—and once again we have failed to confirm their findings.

Dorsal explants of gastrulae do, as they believed, produce CO_2 under anaerobic conditions:

(60) $QanCO_2$ (D,L and R) $= 0.11 \pm 0.04$ (14)

So do ventral explants, though at only a slightly lesser rate:

(61) $QanCO_2$ (V,L and R) $= 0.10 \pm 0.05$ (13)

This small difference between dorsal and ventral pieces, the ratio being:

$$(62)\quad \frac{\text{QanCO}_2\ (\text{D,L and R})}{\text{QanCO}_2\ (\text{V,L and R})} = 1.1 \pm 0.3 \qquad (13)$$

is not statistically significant ($P > 0.05$): thus we must conclude from these results that there is no striking difference between the anaerobic CO_2 output of these two parts of the amphibian gastrula.

Our results have been independently confirmed by Miss Adele Kostellow. With her kind permission her results (at 25° C.) are summarized below:

$$\text{QanCO}_2\ (\text{D,L and R}) = 0.12 \pm 0.04 \qquad (8)$$

$$\text{QanCO}_2\ (\text{V,L and R}) = 0.10 \pm 0.04 \qquad (8)$$

$$\frac{\text{QanCO}_2\ (\text{D,L and R})}{\text{QanCO}_2\ (\text{V,L and R})} = 1.4 \pm 0.7 \qquad (8)$$

As in our experiments, the difference between dorsal and ventral explants is not significant ($P > 0.05$). The high standard deviation (0.7) of her ratios is due to the presence of one experiment in which there was a ratio of 3. If this experiment is deleted, the standard deviation is reduced to 0.2, and the value of the ratio becomes 1.1.

It is not clear to us why our results are at variance with those of Boell *et al.* Perhaps their (apparently unwarranted) correction of CO_2-values by ammonia-values is partly to blame; but among other items which should be considered (and about which information is lacking) are: species-differences (they used *R. temporaria* and *Triton alpestris*); relative stages of gastrulae from which explants were made (we used Stage 10, they used more advanced stages, apparently); duration of anaerobiosis (about five hours for them, about two hours for us); and condition of the explants in the divers (never cytolyzed by interfaces in our experiments; apparently sometimes cytolyzed in theirs; *cf.*, their remarks, p. 353, and Brachet, 1950).

About the last point (explant injury) some comment should be made. In our experiments, the explants were suspended in Holtfreter's solution containing 0.03 *M* bicarbonate (pH after gassing: ca. 7.4), and in this solution the surface-coat of the explants tended to corrode slightly. Visual inspection revealed no apparent cytolysis, but as a confirmatory check on the normality of explants in this solution we obtained their oxygen uptakes, with the result that dorsal explants do indeed respire at a rate significantly different from that of dorsal explants in Holtfreter's without bicarbonate ($P < 0.001$; *cf.* (63) with (1) and (2)), while such ventral explants do not ($P > 0.05$; *cf.* (64) with (3) and (4)).

$$(63)\quad QO_2\ (\text{D,L})\ 0.03\ M\ HCO_3 = 0.27 \pm 0.07 \qquad (7)$$

$$(64)\quad QO_2\ (\text{V,L})\ 0.03\ M\ HCO_3 = 0.25 \pm 0.06 \qquad (7)$$

But, although the relative respiratory rate of dorsal and ventral tissues:

$$(65)\quad \frac{QO_2\ (\text{D,L})\ HCO_3}{QO_2\ (\text{V,L})\ HCO_3} = 1.1 \pm 0.2 \qquad (7)$$

is changed slightly by bicarbonate (($P < 0.001$); (65) compared with (5)), the change is so small that we do not consider it as indicating any crucial actual damage to the tissues.

Summarizing: Contrary to the report of Boell, Needham and Rogers (1939), dorsal and ventral explants do not produce carbon dioxide anaerobically at significantly different rates.

SUMMARY

1. In full-strength Holtfreter's solution, dorsal explants from *Rana pipiens* gastrulae respire some 20% faster than ventral explants from the same gastrulae (with respect to dry weight). Lateral halves of dorsal explants respire at the same rate; and lateral halves of ventral explants respire at the same rate.

2. Azide, cyanide, malonate, p-chloromercuribenzoate, fluoride and 2,4 dinitrophenol in low concentrations all either completely block or greatly retard the gastrulation movements of *Rana pipiens* embryos.

3. Azide, cyanide, malonate, p-chloromercuribenzoate and fluoride all sharply reduce the respiratory rate of both dorsal and ventral explants from 29% (0.0001 M KCN) to 72% (0.001 M NaN_3).

4. 2,4 dinitrophenol (9.5×10^{-6} M) more than doubles the respiratory rate of both dorsal and ventral explants.

5. If these inhibitors block the movements of gastrulation by differential effects upon dorsal and ventral tissues, then these effects are not of such a nature as to be revealed by respiratory measurements—for the relative respiratory rates of dorsal and ventral tissues exposed to inhibitors are the same as those of controls in Holtfreter's solution.

6. Respiratory activity is not linked in any simple way with the movements of gastrulation, for compounds that reduce the former may have no effect upon the latter (0.0005 M KCN), and compounds that may completely block the latter have no effect upon the former (0.0001 M p-chloromercuribenzoate).

7. Dorsal and ventral explants of *Rana pipiens* gastrulae produce carbon dioxide anaerobically, but at rates that are not significantly different.

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THE EFFECTS OF CALCIUM ON ISOLATED ARTHROPOD MUSCLE FIBERS[1]

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The protoplasm of a muscle fiber is normally in a fluid state. This fluidity of muscle protoplasm has been demonstrated in frog muscle by Rieser (1949). Moreover, the protoplasm of muscle like that of other types of cells can and does undergo a gelation—similar to the clotting of blood. For a description of the clotting reaction see Speidel (1938) ; Heilbrunn and Wiercinski (1947) ; Woodward (1948). In marine eggs and protozoa, calcium and thrombin-like substances can cause protoplasm to clot (Heilbrunn and Daugherty, 1932). It is believed that the colloidal system of muscle is essentially similar to the colloidal system of marine eggs and protozoa and, in many respects, is similar to the colloidal system of blood. In an isolated cut muscle fiber the normally fluid protoplasm undergoes a gelation at the cut end of the fiber and this gelation progresses along the fiber (Heilbrunn, 1940). Here then is a system which can be studied. Woodward (1948) has studied the clotting properties of the vertebrate muscle. With few exceptions (for example, see Yaeger and Hager, 1934; Proctor, 1952) there is no available information concerning the effects of calcium on isolated invertebrate muscle fibers. The present study was performed on muscle fibers of invertebrates to determine: 1) whether or not the gelation of the muscle protoplasm is influenced by the calcium ion; 2) to compare the rate of clotting of invertebrate fibers with the rate of clotting of vertebrate fibers; and 3) to determine whether or not the clotting reaction can be inhibited.

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MATERIALS AND METHODS

Various types of arthropods, both terrestrial and marine, were used in the present study of isolated single muscle fibers. The marine forms, which were obtained at the Marine Biological Laboratory (Woods Hole, Mass.), were *Callinectes sapidus* (blue crab) and *Homarus americanus* (lobster). The terrestrial arthropod used mainly in the experiments reported here was *Schistocerca americana* (grasshopper).

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Artificial Ca-free sea water served as the immersion medium for the marine arthropod studies. This modification of McClendon's artificial sea water was made up as follows: 490 cc. of 1 *M* NaCl, 10 cc. of 1 *M* KCl, 30 cc. of 1 *M* $MgCl_2 \cdot 6H_2O$, 20 cc. of 1 *M* $MgSO_4$ and distilled water to make a liter of solution. The pH of this solution varied from 7.8–8.6.

Bělař's (1929) insect Ringer, with calcium chloride omitted, was used as the dissecting medium for studies on the grasshopper.

Merck's reagent grade chemicals and glass (Pyrex) doubly distilled water were used exclusively in the preparation of all solutions.

All pH measurements were made with a glass electrode (Beckman) pH meter. Where necessary, pH adjustments were made with small quantities of 0.1 N NaOH or 0.1 N HCl.

A molar solution of calcium chloride was used as a stock solution and this was diluted with Ca-free Ringer to the desired concentration.

The femur of the large jumping leg (metathoracic) of the grasshopper was removed by cutting just distal to the coxotrochanter and just proximal to the femoral-tibial joint. The chitin was slit both along the anterior and the posterior part of the femur. This reveals the large plume-shaped muscles attached by their relatively large fibers to the chitin of the femur at one end and to a broad flat central tendon at the other. The chitin, which is now in two halves, can be removed by carefully pulling it in opposite directions from the distal end. This process leaves the entire muscle attached to one half of the cuticle. By grasping the muscle by its tendon at its distal end, the entire muscle may be peeled from the cuticle. This muscle appears relatively large, composed of numerous fibers varying in length (1.5–4.0 mm.) and diameter (0.15–0.29 mm.). The entire muscle was placed immediately in Bělař's (Ca-free) insect Ringer and single fibers were isolated by transverse cuts with steel dissecting needles, as close to the tendon as possible and cutting through the numerous tracheoles which bind the fibers.

The ends of the grasshopper muscle fibers, which are fastened to the chitinous exoskeleton, are covered with a brown or white pigment (depending upon the species). This pigmented end does not appear to be very permeable. Thus, the preparation essentially consists of a single muscle fiber each with one cut end. After transferring a fiber to a depression slide with the aid of a pipette, the Ca-free insect Ringer was replaced with a test solution. A fresh cut was made across the fiber and the effects of the test solution were observed immediately. Some authors (see Woodward, 1948) rinse the fibers with two or three changes of the test solution. However, since all the fibers in these experiments were isolated in Ca-free insect Ringer, this technique was not thought necessary.

The clotted portion of the fiber was measured, at varying intervals (30 seconds–30 minutes) after cutting, under a magnification of approximately 43 ×, by means of a Ramsden-type filar micrometer eyepiece (Bausch and Lomb with 12.5 × eyepiece).

Muscle fibers of marine forms (crab and lobster) were isolated by cutting off the walking legs, removing the chitinous covering, and dissecting out single fibers from the exposed muscles in Ca-free sea water. In no cases were fibers used experimentally more than 45 minutes after amputation of the leg from the animal.

Results

Under the influence of calcium two visible changes occur in isolated single muscle fibers of arthropods: 1) a shortening of the fiber, and 2) the formation of a clot. Although these two phenomena occur concurrently in invertebrate muscle fibers, they will be considered separately in this paper.

1. *Shortening*

a. Blue crab. Single muscle fibers from the blue crab (*C. sapidus*), which had been isolated in Ca-free sea water, were placed immediately in isotonic calcium chloride solution (0.33 *M*). In each case, a fresh cut was made across the fiber and the length of the cut single fiber was then measured. As indicated by Tables I and II, these pieces of isolated muscle fiber varied in their original length. Following exposure to calcium the fibers shorten. The degree of shortening of each fiber was determined by the product of the final length times 100, divided by the

Table I

Effects of isotonic $CaCl_2$ *(0.33 M) pH 5.7–6.0 (single muscle fibers)*

Blue Crab (*Callinectes sapidus*)

Original length mm.	Time min.	Rate of clotting mm./min.	Final length mm.	% Shortened
3.519	1.0	0.247	1.711	51.3
2.608	1.0	0.219	1.556	59.6
3.651	1.0	0.293	2.934	19.7
3.995	1.0	0.472	1.980	50.5
3.504	1.0	0.446	1.801	48.7
3.463	1.0	0.352	2.078	40.0
3.569	1.0	0.802	2.475	30.4
3.341	1.0	0.207	1.548	53.7
3.260	1.0	0.393	1.483	54.5
5.623	1.0	0.262	2.863	48.6
3.260	1.0	0.328	2.773	14.0
2.950	0.75	0.958	2.078	29.6
3.039	1.0	0.413	1.752	42.4
3.260	1.0	0.195	2.387	26.8
2.542	0.5	1.03	1.418	44.3
2.852	1.0	0.250	1.344	52.9
2.811	0.25	0.995	1.956	30.5
2.991	1.0	0.351	1.589	49.6
3.121	1.0	0.487	2.094	33.0
2.469	1.0	0.387	1.548	37.3
3.285	1.0	0.359	2.135	34.1
4.792	1.0	0.328	1.996	58.4
3.537	1.0	0.767	2.942	16.9
		0.461		40.3

TABLE II

Effects of isotonic $CaCl_2$ (0.33 M) pH 8.0–8.2 (single muscle fibers)

Blue Crab (*Callinectes sapidus*)

Original length mm.	Time min.	Rate of clotting mm./min.	Final length mm.	% Shortened
4.360	1.0	0.588	1.654	62
3.243	1.0	—	1.670	48.5
5.321	1.0	0.608	1.956	63.3
5.599	1.0	0.328	3.553	36.6
5.028	1.0	0.467	3.088	38.6
3.349	1.0	0.640	2.326	31.2
8.581	1.0	0.452	4.360	49.2
3.594	0.5	0.502	1.793	50.2
7.359	1.0	0.502	2.591	64.8
7.726	1.0	0.410	5.028	35.0
8.655	1.0	0.443	5.868	32.3
6.414	1.0	0.561	3.742	41.7
6.715	2.0	0.294	4.319	35.7
		0.482		45.3

original length. This value when subtracted from 100 gives the per cent of shortening. This may be stated:

$$\text{per cent of shortening (p. s.)} = 100 - \left(\frac{\text{final length}}{\text{original length}} \times 100\right)$$

The shortening of fibers exposed to 0.33 M $CaCl_2$ (pH 5.7 to 6.0) varied from 14% to 59% (Table I). The average per cent shortening was about 40%. The pH of this solution (5.7–6.0) was lower than that of normal sea water. When isolated blue crab muscle fibers were exposed to 0.33 M $CaCl_2$ at the normal pH of sea water (8.0–8.2), the average shortening was 45.3% (Table II).

b. Lobster. Twelve fibers from the lobster (*H. americanus*), immersed in 0.33 M $CaCl_2$, showed an average shortening of 33.7%.

c. Grasshopper. The fibers of the grasshopper (*S. americana*) were extremely sensitive to calcium. Twenty-three fibers of this arthropod showed an average shortening of 58% in a solution containing calcium chloride in a concentration of 0.0018 M.

2. *Clotting*

The second visible change occurring in isolated single muscle fibers, as a result of the action of the calcium ion, is the formation of a clot. The clotted protoplasm appears only at the cut end or other point of injury of the muscle fiber. The area of the clot appears as a swollen region darker in color than the remaining protoplasm. This clot moves as a wave along the muscle fiber. In addition, the clotted portion is characterized by a loss of cross striations.

a. Rate of clotting.

1) Blue crab and lobster. Data were gathered at one-minute intervals to determine the rate of movement of the protoplasmic clot in isolated single muscle fibers of the blue crab under the influence of various concentrations of calcium. The effects of four different concentrations on blue crab (*C. sapidus*) are summarized in Figure 1. In all concentrations of calcium observed, there was a relatively high rate of movement of the protoplasmic clot of the muscle fibers during the first minute of exposure. However, this rate was not maintained at this high level for the entire period of exposure of these muscles in calcium. In Figure 1 the ordinates show the length of the clotted material in the fiber and the abscissae the time. The rates at which the clotted portion of the fiber increases in length are given by the slopes of the curves. In artificial sea water (0.0117 *M* $CaCl_2$), the average rate of movement of the protoplasmic clot of 10 fibers was 0.287 mm./min. at one minute and decreased to a rate of 0.108 mm./min. at 4 minutes (see Fig. 1). The average rate of movement of the protoplasmic clot of 5 fibers exposed

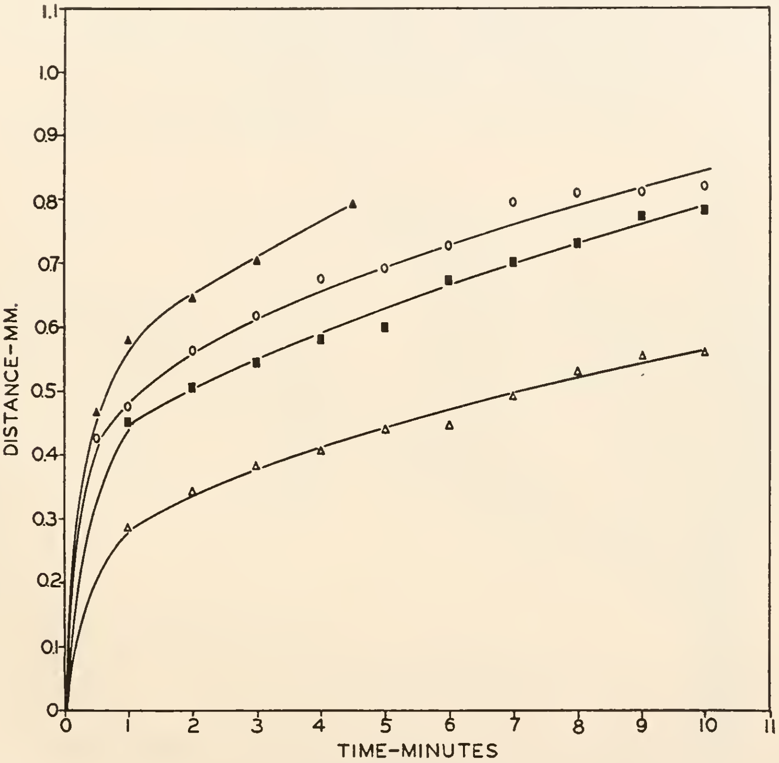

FIGURE 1. The effects of calcium on single muscle fibers of the blue crab (*Callinectes sapidus*). Legend: Open triangles (△), artificial sea water, pH 7.8, average of 10 fibers; solid squares (■), 0.083 *M* $CaCl_2$ in artificial Ca-free sea water, pH 8.2, average of 5 fibers; open circles (○), 0.165 *M* $CaCl_2$ in artificial Ca-free sea water, pH 7.5, average of 13 fibers; solid triangle (▲), 0.330 *M* $CaCl_2$ (pure), pH 6.89, average of 10 fibers.

to 0.083 *M* $CaCl_2$ was 0.451 mm./min. at one minute and decreased to 0.075 mm./min. at 10 minutes. Thirteen fibers exposed to a concentration of 0.165 *M* $CaCl_2$ showed an average rate of clotting of 0.475 mm./min. at one minute and 0.0824 mm./min. at 10 minutes. Isotonic calcium chloride solution (0.33 *M*) gave a value (average of 10 fibers) of 0.534 mm./min. at one minute and 0.176 mm./min. at 4.5 minutes (see Fig. 1).

Attempts to obtain single muscle fibers from the lobster (*H. americanus*) suitable for a study of the rate of clotting were unsuccessful. These fibers showed an inherent tendency to stick together. This "stickiness" of the fibers caused them to adhere to the dissecting needles, thus causing injury.

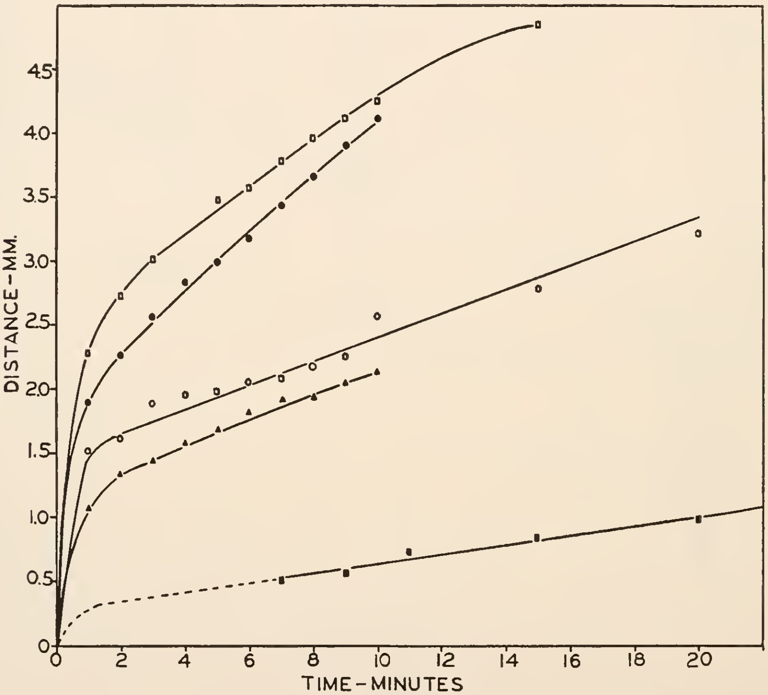

FIGURE 2. The effects of calcium on single muscle fibers of the grasshopper (*Schistocerca americana*). Legend: Open squares (□), 0.0175 *M* $CaCl_2$ in Ca-free Ringer, pH 7.25, average of three fibers; solid circles (●), 0.0087 *M* $CaCl_2$ in Ca-free ringer, pH 7.22, average of three fibers; open circles (○), 0.0044 *M* $CaCl_2$ in Ca-free Ringer, pH 7.32, average of 6 fibers; solid triangles (▲), 0.0018 *M* $CaCl_2$ in Ca-free Ringer, pH 7.5, average of 9 fibers; solid squares (■), Ca-free Ringer, pH 7.3, average of 5 fibers.

2) Grasshopper. Data were gathered at one-minute intervals to determine the rate of clotting of protoplasm in various concentrations of calcium. The wave-like movement of the clot along the muscle fiber showed considerable variation in speed even in fibers from the same animal.

Figure 2 summarizes the results of the effects of various concentrations of calcium on single muscle fibers of the grasshopper (*S. americana*). The ordinates show the length of the clotted material in the fiber, the abscissae the time. The rates at which the clotted material increases in length are given by the slopes of the curves. Four concentrations of calcium chloride were used in these experiments (see Fig. 2) : 0.0175 *M* (top curve, open squares) ; 0.0087 *M* (solid circles) ; 0.0044 *M* (open circles) ; and 0.0018 *M* (solid triangles). The pH of these test solutions varied from 7.22–7.50. Muscle fibers exposed to these four test solutions show a relatively high rate of movement of the protoplasmic clot during the first minute of exposure. The effects of various concentrations of calcium on the rate of protoplasmic clotting of single muscle fibers during the first minute are as follows: fibers immersed in 0.0175 *M* $CaCl_2$ showed an average rate of clotting of 2.297 mm./min.; fibers immersed in 0.0087 *M* $CaCl_2$ had an average clotting rate of 1.899 mm./min.; fibers immersed in 0.0044 *M* $CaCl_2$ showed an average clotting rate of 1.512 mm./min.; and those immersed in 0.0018 *M* $CaCl_2$ (the calcium concentration normally present in insect Ringer) had an average rate of clotting of 1.050 mm./min. This relatively high rate of movement of the clot is not maintained during the later stages of the clotting process (see Fig. 2). Thus, the rate of clotting of fibers exposed to 0.0018 *M* $CaCl_2$ had an average clotting rate of 0.332 mm./min. at 5 minutes and 0.211 mm./min. at 10 minutes. Fibers exposed to 0.0044 *M* $CaCl_2$ showed an average rate of clotting of 0.394 mm./min. at 5 minutes and 0.234 mm./min. at 10 minutes. Those fibers immersed in 0.087 *M* $CaCl_2$ and those immersed in 0.0175 *M* $CaCl_2$ had an average rate of clotting of 0.596 mm./min. and 0.696 mm./min. at 5 minutes and 0.410 mm./min. and 0.425 mm./min. at 10 minutes, respectively.

TABLE III

Effects of calcium on rate of protoplasmic clotting (single muscle fibers)

Grasshopper (*Schistocerca americana*)

Ringer (.0018 *M* $CaCl_2$) mm./min.	Ca-free Ringer at 7 min. mm.	2.5 × conc. Ca (.0044 *M*) in Ringer mm./min.	10 × conc. Ca (.0175 *M*) in Ringer mm./min.	K oxalate (.005 *M*) in Ca-free Ringer after 15 min. mm.	Na oxalate (.005 *M*) in Ca-free Ringer after 15 min. mm.
1.281	0.536	1.616	2.315	0	0
1.144	0.526	1.498	2.143	0	0
1.053	1.135	1.798	2.433	0	0
0.962	0.490	1.398		0	0
1.262		1.253		0	0
0.944				0	0
0.726				0	0
1.088					
0.971					
1.050	0.671	1.512	2.297		

Figures show rate at one minute unless otherwise indicated.

The rate of movement of the protoplasmic clot of grasshopper muscle fibers in Ca-free Ringer is difficult to determine during the first few minutes of observation. These difficulties are encountered only during the first 6–7 minutes. This period is characterized by a gradual darkening of the protoplasm in the area adjacent to the cut or exposed end. There is no definite boundary separating the clotted region from the unclotted portion of the fiber. However, after 7 minutes, in all cases, there was a distinct clotted portion with a definite boundary characteristic of fibers exposed to higher concentrations of the calcium ion. In Figure 2 the time during which there is indistinct clotting is represented by a broken line. The average rate of clotting of 5 fibers at 7 minutes was 0.072 mm./min. and 0.066 mm./min. at 11 minutes.

b. Inhibition of clotting. It has been shown that the rate of movement of the protoplasmic clot in muscle fibers is dependent upon the calcium concentration. The question now arises as to whether or not the clot progresses in the absence of calcium.

1) Blue crab

With muscle fibers from the blue crab (*C. sapidus*), it was unnecessary to use sodium or potassium oxalate to prevent the movement of the protoplasmic clot. Fibers dissected and cut in Ca-free sea water and allowed to remain in this medium for periods of from 20–36 minutes showed no evidence of protoplasmic clotting during this period of exposure (see Table IV). Upon return to artificial sea water containing a concentration of calcium of 0.11 *M*, these same fibers immediately showed the clotting reaction characteristic of muscle exposed to calcium.

TABLE IV

Effects of calcium on rate of protoplasmic clotting (*single muscle fibers*)

Blue Crab (*Callinectes sapidus*)

Artificial sea water (0.0117 *M* $CaCl_2$) mm./min.	Isotonic $CaCl_2$ (.33 *M*) mm./min.	1/2 isotonic $CaCl_2$ (.165 *M*) mm./min.	Ca-free sea water (from 20–36 min.) mm./min.
0.287	0.586	0.448	0
0.362	0.513	0.456	0
0.382	0.578	0.619	0
0.225	0.448	0.464	0
0.327	0.741	0.619	0
0.265	0.448	0.448	0
0.205	0.432	0.456	0
	0.603	0.513	0
	0.472	0.554	0
0.293	0.534	0.508	

2) Grasshopper

In the absence of calcium from the surrounding medium, the protoplasmic clot progresses. Thus, if a single muscle fiber from the grasshopper is isolated in Ca-free insect Ringer, a protoplasmic clot will form and progress (see bottom curve, Fig. 2). However, either sodium or potassium oxalate when mixed with Ca-free insect Ringer is effective in preventing the formation of a clot (see Table III).

The concentration of potassium (or sodium) oxalate which was most effective with grasshopper muscle fibers was 0.005 *M*. Higher concentrations usually resulted in death of the muscle fibers, whereas lower concentrations were not effective in preventing the movement of the clot. Figure 3 summarizes the results of experiments with potassium oxalate in inhibiting the rate of movement of the protoplasmic clot. A series of experiments was performed in which muscle fibers of the grasshopper (*S. americana*) were cut in a K-oxalate (0.005 *M*)-Ca-free insect Ringer solution and allowed to remain in this solution for 15 minutes. During this period, no protoplasmic clot could be observed in these fibers (see bottom curve, Fig. 3). However, after the 15-minute period had expired, each muscle fiber was placed in normal insect Ringer. There was an immediate clotting of the muscle protoplasm. The rate of movement of this protoplasmic clot was 1.245 mm./min. during the first minute and 2.040 mm./min. at the end of 5 minutes (average of 5 fibers). These experiments clearly demonstrate the inhibitory effect of K-oxalate on the movement of the clot in muscle protoplasm. The effectiveness of both potassium and sodium oxalate is shown in Table III.

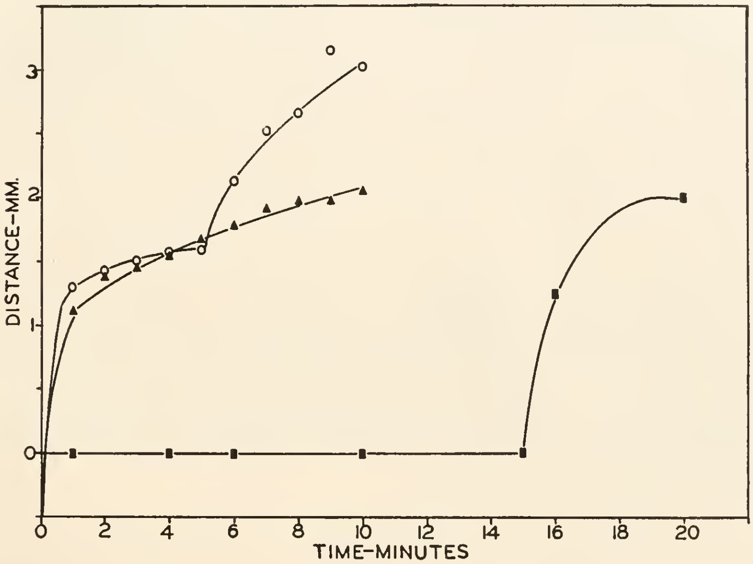

FIGURE 3. The effects of oxalate on protoplasmic clotting in single muscle fibers of the grasshopper (*Schistocerca americana*). Legend: Open circles (○), Ringer for one minute, K-oxalate (0.005 *M*) for the next 4 minutes, Ringer for 5 minutes, average of 6 fibers; triangles (▲), control, Ringer for 10 minutes, average of 5 fibers; squares (■), K-oxalate (0.005 *M*) for 15 minutes, Ringer for 5 minutes, average of 5 fibers.

Another experiment was performed to determine whether this clotting reaction, once initiated by calcium, could be stopped. In this experiment a muscle fiber was first exposed to normal insect Ringer (containing calcium as the clotting agent) for a period of time sufficient to initiate the clotting reaction. Then the fiber was washed and the Ringer solution replaced with an oxalate-Ringer solution. After observations were made on the progress of the clot, the oxalate-Ringer solution was replaced with normal insect Ringer and the rate of clotting was again observed. The results of this experiment are shown in Figure 3 (top curve). A detailed account of this experiment follows. Muscle fibers were cut and allowed to remain in normal insect Ringer (calcium concentration, 0.0018 *M*) for one minute (see Fig. 3, top curve). During this period the average (6 fibers) rate of movement of the clot was 1.300 mm./min. These fibers were then quickly washed three times with oxalate-Ringer (0.005 *M* K-oxalate in Ca-free insect Ringer) and allowed to remain in this solution for four minutes. The average rate of movement of the clot of these 6 fibers was determined at one-minute intervals during exposure to the oxalate-Ringer solution. In Figure 3 (top curve) the point at one minute indicates the distance the clot had progressed during a one-minute exposure to normal insect Ringer. The next four points (2, 3, 4 and 5 minutes) indicate the progress of the clot under the influence of oxalate-Ringer for 4 minutes. The average rates of movement of the protoplasmic clot while immersed in oxalate-Ringer were: 1.432 mm./min. during the first minute; 0.750 mm./min. during the second minute; 0.525 mm./min. for the third minute; and 0.936 mm./min. during the fourth minute. These fibers were then washed with normal insect Ringer and the rate of movement of the protoplasmic clot was determined at one minute intervals in insect Ringer. As indicated by the curve (Fig. 3, top curve), exposure of these previously oxalated fibers to a solution containing calcium (normal insect Ringer) results in a rapid increase in the rate of movement of the protoplasmic clot. The average rates of movement of the clot during a 5-minute exposure to normal insect Ringer were: 2.147 mm./min., 1.261 mm./min., 0.888 mm./min., 0.789 mm./min., and 0.606 mm./min., respectively.

Six control fibers, cut and allowed to remain in normal insect Ringer for 10 minutes, gave the following average rates of movement of the clot: during the first minute, 1.071 mm./min.; second minute, 0.673 mm./min.; third minute, 0.483 mm./min.; fourth minute, 0.396 mm./min.; fifth minute, 0.339 mm./min.; sixth minute, 0.302 mm./min.; seventh minute, 0.275 mm./min.; eighth minute, 0.243 mm./min.; ninth minute, 0.229 mm./min.; and tenth minute, 0.214 mm./min. (see triangles, middle curve, Fig. 3).

Discussion

Of the four major cations (Na^+, K^+, Mg^{++} and Ca^{++}) normally present in protoplasm, the calcium ion has long been known to be of primary importance in physiological processes. Its importance has been demonstrated in the clotting of blood and milk, the surface precipitation reaction of various cells, and more recently in relation to the stimulation in muscle. In fact, Heilbrunn has proposed a theory of stimulation and anesthesia based on the presence of calcium in the cortex of the cell and its subsequent movement to the interior. For a discussion of this theory and the relation of calcium to these phenomena see Heilbrunn (1943) and Woodward (1948). In order to study the effect of the calcium ion on muscle, physiologists have used isolated muscle fibers of vertebrates. The data in this paper reveal some of the similarities between vertebrate and invertebrate muscle fibers in their response to calcium.

One of the first visible effects of the calcium ion on vertebrate (frog) muscle fibers is a marked shortening of the fiber (Heilbrunn, 1940). In studies on isolated muscle fibers of three arthropods (blue crab, lobster and grasshopper) it was found that generally these forms follow the same pattern as vertebrate isolated muscle fibers. In the case of the lobster and the blue crab the fibers shortened to a lesser degree than isolated fibers from the frog in comparable solutions of calcium chloride. Thus, with frog fibers Heilbrunn found an average shortening of 73%.

The average percentage of shortening in muscle fibers of the blue crab was about 45% and in fibers of the lobster about 34%. Normal insect Ringer with a calcium concentration of 0.0018 *M* caused an average percentage of shortening of 58% in grasshopper muscle fibers. The evidence presented indicates that the sensitivity of muscle fibers of these forms to calcium is dependent upon the amount of calcium normally present in their environment.

A second visible effect of calcium on arthropod muscle fibers was the clotting reaction reported earlier by Speidel (1938), Heilbrunn and Wiercinski (1947) and Woodward (1948). The data show that this protoplasmic clotting in arthropod muscle is quite similar to that found by other investigators in vertebrate (frog) muscle fibers. However, the rate at which the movement of the protoplasmic clot occurs in the grasshopper indicates that the muscles of this animal are much more sensitive to low concentrations of calcium than those of the frog or the blue crab. Electrical stimulation has been used as a criterion for determining whether single muscle fibers of the frog are viable. With arthropod single muscle fibers, this method of determining their viability was found to be unreliable. A convenient indication of the viability of these fibers is the effect produced by the calcium ion—the clotting reaction.

It is obvious, therefore, that the effect of calcium on muscle protoplasm is much the same for muscle fibers of widely different types of animals. In frog muscle, however, it is difficult or impossible to prevent shortening and the clotting reaction by removing calcium from the surrounding medium. In all the invertebrate muscles I have studied, removal of calcium from the surrounding medium prevents shortening and clotting. This fact adds additional support to Heilbrunn's point of view, for it shows a complete dependence of this clotting of muscle protoplasm on the calcium ion.

Experiments showed that, once initiated, this clotting reaction, particularly in the grasshopper, does not stop immediately. There is a gradual decrease in the speed of movement of the clot in oxalate-Ringer solution. The data presented (Fig. 3) show a significant difference between the rate of movement of the clot in oxalate-Ringer solution as compared to normal insect Ringer. This suggests that not only is this clotting reaction a calcium-initiated phenomenon, but also that it requires calcium for its continuance.

SUMMARY

1. When isolated single muscle fibers of blue crab, lobster and grasshopper have their cut ends exposed to solutions containing the calcium ion, they show the same effects as do frog muscle fibers; that is to say, they shorten and the protoplasm clots. However, the wave of clotting is faster in grasshopper muscle than it is in frog muscle. The degree of shortening of arthropod muscle fibers is less than that of frog muscle.

2. Evidence indicates that single muscle fibers of the grasshopper are more sensitive to calcium ions than are single fibers of the frog and blue crab.

3. The rate of movement of the protoplasmic clot in invertebrates is relatively rapid during the first minute. However, there is a gradual decrease in this rate after the first minute.

4. The clotting reaction can be prevented in invertebrate muscle fibers. In grasshopper muscle fibers, concentrations of 0.005 *M* sodium or potassium oxalate are effective in preventing the formation of the protoplasmic clot. Ca-free sea water is in itself sufficient to prevent the formation of the protoplasmic clot in muscle fibers of the blue crab. Once initiated, this clotting reaction in crab muscle cannot be stopped immediately by the application of sodium or potassium oxalate.

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SEROLOGICAL COMPARISONS OF ASTACURAN CRUSTACEA[1]

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The methods of comparative serology have shown that the proteins of the sera of organisms are stable systems. These proteins are inherited in relatively simple ways and are not modified to any appreciable degree by environmental influences. Therefore such proteins can be considered in a natural scheme of classification. The purpose of the study here reported upon is to make serological comparisons of the serum proteins (the hemocyanins) of astacuran Crustacea. Eleven species from three families (Astacidae, Parastacidae and Homaridae) are studied.

Review of Literature

Earlier systematic serologists used various modifications of precipitin testing to establish relationships; only a few of the studies were quantitative. Within the past decade quantitative serological techniques have been developed to estimate the taxonomic relationships among animals.

Comparative serology of long-tailed Crustacea began with the studies of Nuttall (1904) and Graham-Smith (*in* Nuttall), both of whom used the same antiserum produced against the serum of *Homarus vulgaris* Milne-Edwards [= *Homarus gammarus* (Linnaeus)]. When reacted against a variety of other animal sera, the anti-*Homarus vulgaris* immune serum [= *H. gammarus*] reacted only with its homologous antigen and with the antigens of other decapods. As a result of these studies both Nuttall and Graham-Smith held that the degree of reaction between anti-*Homarus vulgaris* serum [= *H. gammarus*] and proteins of a variety of species was in general proportional to the amount of relationship of these proteins to each other. Their results, obtained from serological tests with animal bloods, did confirm, in general, the accepted classification of these animals which was based chiefly on comparative anatomy, embryology and paleontology.

Erhardt (1929), following some modifications of the methods used by Nuttall and by Graham-Smith, produced an antiserum against the serum of *Potamobius astacus* Linnaeus [= *Astacus astacus* (Linnaeus)], and reacted it against the homologous serum plus the sera of *Potamobius leptodactylus* Linnaeus [= *Astacus leptodactylus* Eschscholtz], *Homarus vulgaris* [= *Homarus gammarus*], several brachyuran species and numerous other invertebrates. The results of these experiments agreed comparatively well with the classification of these animals in that the sera of crayfish belonging to the family Astacidae reacted positively with the anti-*Potamobius astacus* serum [= *Astacus astacus*] whereas the sera of other decapods and schizopods gave negative results. Erhardt did not, however, discover the value of employing antigens of known protein concentrations. The antigens studied in these experiments were arbitrarily diluted from 1 : 8 to 1 : 256 in a doubling-dilution series and tested with the anti-*Potamobius astacus* serum [= *Astacus astacus*]. Erhardt observed also that the antisera resulting from his injections of crustacean sera into rabbits were compounded of a series of partial precipitins which did not always develop in an equivalent manner in different rabbits exposed to the same antigen.

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[1] These studies were aided by a contract (NR 162–013) between the University of Kansas and the office of Naval Research, Department of the Navy.

Clark and Burnet (1942) reported the separation of the various genera of Parastacidae from each other and from other Decapoda, by using the ring-test precipitin reaction, and absorption tests. Of the astacuran Crustacea, the hemocyanins of *Euastacus yarraensis* (McCoy), *Euastacus armatus* (von Martens), *Euastacus nobilis* (Dana), *Euastacus bispinosus* Clark, *Cherax albidus* Clark, *Cherax destructor* Clark, *Cherax rotundus* Clark, *Cherax bicarinatus* (Gray), *Cherax quinquecarinatus* (Gray), *Engaeus fultoni* Smith and Schuster and *Engaeus victoriensis* Smith and Schuster, were studied. The results obtained by Clark and Burnet indicated (1) the existence of several completely distinct "antigenic types" (*op. cit.,* p. 94) within the Decapoda, (2) the value of absorption experiments in studying close relationships, and (3) that hemocyanin was the only antigen in the serum of *Jasus lalandii* (Lamarck), a palinuran crustacean.

Boyden (1943) was the first to use a quantitative technique to obtain serological data for the determination of taxonomic relationships of long-tailed Crustacea. The degree of relationship between *Homarus americanus* Milne-Edwards and *Homarus vulgaris* Milne-Edwards [= *Homarus gammarus* (Linnaeus)] was determined by using a turbidimetric analysis of precipitates covering the whole range of reactivity between antigen and antiserum. Boyden concluded that the serological relationship of these species agreed with their generally accepted systematic positions, and that *Homarus americanus* and *Homarus vulgaris* [= *Homarus gammarus*] are valid species.

Leone (1950) reported further quantitative serological data from tests of some nephropsidean and astacuran Crustacea. Hemocyanins from *Homarus americanus* Milne-Edwards, *Homarus vulgaris* Milne-Edwards [= *Homarus gammarus* (Linnaeus)], *Cambarus clarkii* Girard [= *Procambarus clarkii* (Girard)], *Euastacus elongatus* Clark, *Euastacus nobilis* (Dana), *Euastacus armatus* (von Martens), *Cherax destructor* Clark and *Cherax albidus* Clark, were compared. The degree of relationship between the two species of *Homarus* agreed with the findings of Boyden in that they showed a high degree of serological correspondence. Considering only the serological data, Leone questioned whether the difference between them is sufficient to merit specific status for each. Leone concluded that *Euastacus elongatus*, *Euastacus nobilis* and *Euastacus armatus* exhibit a high degree of serological correspondence and are probably the same or closely related species. This conclusion confirms the findings of Clark and Burnet that *E. armatus* and *E. nobilis* are more closely related than either is to *E. sulcatus* or *E. bispinosus*. Leone further concluded that 20-minute reaction times for precipitin reactions established significant orders of relationships among animal sera. Twenty-four hour reaction time does not alter the order of relationship from that established at 20 minutes despite considerable increases in amounts of reaction recorded.

Leone and Pryor (1952) made serological comparisons of saline-hemocyanin filtrates of three species of shrimp, *Penaeus aztecus* Ives, *Penaeus setiferus* (Linnaeus) and *Penaeus duorarum* Burkenroad, using a photoelectric turbidimeter. These workers concluded that *P. aztecus* and *P. duorarum* are more closely related to each other than either is to *P. setiferus*. Their conclusion is in accord with Burkenroad's morphological arrangement of the three species (Burkenroad, 1949).

Materials and Methods

Antigens

Hemocyanin is the single protein in the sera of Crustacea (Allison and Cole, 1940; Clark and Burnet, 1942; Tyler and Scheer, 1945). This protein is an excellent antigen. The hemocyanins studied by us have been prepared with a minimum amount of preparatory treatment so that from physical handling, little or no chemical change occurred to alter biochemical characters. A list of the Crustacea from which the antigens were prepared is found in Table I. The taxonomic positions of species of the family Astacidae given in the Table follow those of Williams and Leonard (1952); the classification of species in the family Parastacidae is that of Hale (1927).

Kansas crayfishes

Approximately 50 specimens each of *Orconectes immunis* (Hagen), *Orconectes nais* (Faxon), *Orconectes neglectus* (Faxon) and *Procambarus simulans* (Faxon) were collected from ponds and streams in the state of Kansas and identified by the authors and by Dr. Austin B. Williams. As the crayfishes were received in the laboratory they were starved for 12 to 18 hours prior to bleeding, to remove the food materials that may have been present in the circulatory system and which might influence the true biochemical characteristics of the hemocyanins.

To obtain hemocyanins from the crayfishes they were bled by use of a 22 gauge needle and a 10 cc. syringe. A needle was inserted cephalad into the ventral abdominal artery and the blood drawn directly into the syringe. For each lot of crayfish of a given species, the bloods were pooled and placed in clean test tubes, allowed to clot, centrifuged at 2000 rpm for one-half to one hour, and the serum decanted. There remained after centrifugation a thick jelly-like clot which was wrapped in double thicknesses of fine cheese cloth and twisted manually to express the serum still remaining (Leone, 1949). These methods yielded a light bluish-green serum which was sterilized by filtering with Seitz filters, bottled in sterile five-cc. vials and stored at 0° ± 2° C., until needed for testing.

Orconectes virilis

Twenty-four specimens of *Orconectes virilis* (Hagen) were collected from Lake Winnebago, Wisconsin, and identified by Dr. A. Byron Leonard.

Hemocyanins from these crayfish were obtained by use of the procedure given for the Kansas crayfishes.

Table I

A list of species, the sera of which were used in the study of the relationships among astacuran Crustacea, and injection schedules of these sera used to produce antisera

Species	Serum protein gms./100 cc.	Injection schedule	Series of injections
Tribe Astacura			
Family Astacidae			
Procambarus simulans (Faxon)	2.50	0.25 cc., subcut., 0.1 cc., 0.2 cc., 0.5 cc., IV	1
Orconectes neglectus (Faxon)	3.00	0.25 cc., subcut., 0.1 cc., 0.2 cc., 0.5 cc., IV	1
Orconectes nais (Faxon)	8.00	0.25 cc., subcut., 0.1 cc., 0.2 cc., 0.5 cc., IV	1
Orconectes immunis (Hagen)	8.70	0.25 cc., subcut., 0.1 cc., 0.2 cc., 0.5 cc., IV	1
Orconectes virilis (Hagen)	5.65	0.25 cc., 0.5 cc., 0.75 cc., 1.0 cc., IV	2
Family Parastacidae			
Euastacus elongatus Clark	6.95	0.25 cc., 0.5 cc., 0.75 cc., 1.0 cc., IV	2
Euastacus nobilis (Dana)	3.90	0.25 cc., 0.5 cc., 0.75 cc., 1.0 cc., IV	2
Euastacus armatus (von Martens)	3.05	0.25 cc., 0.5 cc., 0.75 cc., 1.0 cc., IV	2
Cherax albidus Clark	5.40	0.25 cc., 0.5 cc., 0.75 cc., 1.0 cc., IV	2
Cherax destructor Clark	5.25	0.25 cc., 0.5 cc., 0.75 cc., 1.0 cc., IV	2
Family Homaridae			
Homarus americanus Milne-Edwards	3.35	0.5 cc., 0.5 cc., 0.5 cc., subcut.	3

Freshwater crayfishes from Australia

The sera from single specimens of *Cherax albidus* Clark, *Cherax destructor* Clark, *Euastacus armatus* (von Martens), *Euastacus elongatus* Clark, and *Euastacus nobilis* (Dana) were obtained from the collection of invertebrate sera at the University of Kansas. Originally these samples were prepared by Miss Ellen Clark, who collected the crayfishes from Broken Creek, Victoria, Australia. The same procedure as outlined for the Kansan crayfishes was used for the preparation of hemocyanins from the Australian species, with one exception: to each 9.0 cc. of serum was added 1.0 cc. of two per cent formalin solution which served as a bacteriostatic agent.

Homarus americanus

One of us (Leone) collected the hemocyanin from one specimen of *Homarus americanus* netted from the North Atlantic Ocean off the coast of Cape Cod, Massachusetts. A slit was made through the ventral hemocoel, at the junction of the cephalothorax and the abdomen, from which the animal was permitted to bleed through a clean, glass funnel into suitable containers. Whole blood obtained by this procedure was allowed to clot, and then centrifuged to hasten the expression of serum. "Merthiolate" in a dilution of 1 : 5,000 was added to the serum as a bacteriostatic agent.

Contamination of hemocyanin preparations

Some of the vials of hemocyanin preparations stored without preservatives became contaminated with bacteria despite the care exercised during filtration and bottling, but because of the stability of these proteins such contamination probably did not cause appreciable alteration. Leone (1949) observed that such contaminated hemocyanins stored over short periods at 0° ± 2° C. showed no detectable serological alterations after re-sterilization through Seitz filters.

Antisera

All antisera were produced in healthy, adult rabbits. A list of the antigens used to produce these antisera and their injection schedule is given in Table I. For the antisera to the proteins of Kansas crayfishes a pre-sensitizing dose of 0.25 cc. of antigen was injected subcutaneously, and the animal was permitted to rest for 30 days; each rabbit then received a single series of intravenous injections: 0.1 cc., 0.2 cc., and 0.5 cc., on alternate days. Rabbits producing the anti-*Homarus americanus* serum received three series of subcutaneous injections, each consisting of 0.5 cc., 0.5 cc., and 0.5 cc., on alternate days. The other antisera were produced by giving the rabbits two series of injections; a single series consisted of 0.25 cc., 0.5 cc., 0.75 cc., and 1.0 cc., given intravenously on alternate days.

Trial bleedings of the rabbits were made 8 days after the last injection of a series by taking whole blood from the median artery of the ear, while partial bleedings were made by direct cardiac puncture. For bleeding from the ear a 22 gauge hypodermic needle and a 10 cc. syringe were used. If, after either method of bleeding, there were sufficient antibodies in the serum for serological testing, a terminal bleeding was performed by direct cardiac puncture.

The whole blood obtained by the above methods was placed in clean test tubes and allowed to clot, after which it was rimmed, then placed at 0° ± 2° C. for 12 to 18 hours; the serum containing antibodies was then decanted. This antiserum was centrifuged to clear it of all blood cells, sterilized in Seitz filters, bottled in sterile vials, and stored at 0° ± 2° C. until used.

Methods of Testing

The methods of precipitin testing used in this study follow those outlined by Boyden and DeFalco (1943), and Leone (1949). Measurements of all turbidities were made with the Libby (1938) photronreflectometer. Critical analyses of the performance of the Libby photronreflectometer have been made (Boyden and DeFalco, 1943; Boyden, Bolton and Gemeroy, 1947; Bolton, Leone and Boyden, 1948). These authors conclude that for white precipitate systems, which include all the precipitin reactions, this instrument is valuable and reliable in studying the characteristics of such precipitates.

Precipitin tests were made by adding a constant amount of antiserum to doubling dilutions of antigens. The antigens were diluted with 0.9 per cent saline, buffered with $M/150$ phosphate salts (Evans, 1922). Tests were run in standard

Kolmer test-tube racks with a capacity of 48 tubes. A single test consisted of 17 Kolmer tubes into which was placed 1.7 cc. of diluted antigen; the first tube contained the initial dilution and each successive tube contained a protein dilution one-half the concentration of the preceding tube, ranging from 1 : 62.5 to 1 : 4,096,000. These dilutions were precisely made on the basis of the known protein concentration of the undiluted serum (Table I). Two control tubes were always used: a saline control, and an antiserum control which contained 1.7 cc. of the saline solution plus 0.3 cc. of antiserum. "Merthiolate" in a final dilution of 1 : 10,000 was added to prevent bacterial growth which would otherwise influence measurements of turbidities.

An antigen turbidity control value was obtained for each antigen dilution by transferring this solution to the cell of the photronreflectometer, and the amount of light reflected due to the cell itself plus its contents was then recorded and the dilution poured back into the Kolmer tubes. Readings for the antiserum and saline controls were likewise recorded. After the control readings for each dilution of antigen had been made, 0.3 cc. of antiserum was added and the tube placed in the dry air incubator maintained at 37° C. After 20 minutes, turbidities resulting from the antigen-antibody reactions were measured and recorded. Each tube was then stoppered with a cork to prevent excessive evaporation and left at room temperature for 24 hours, after which time readings were again made of the turbidities.

The control turbidities for the antigen and the antiserum were deducted from the total turbidity developed in any tube. The resultant values were assumed to be due to the precipitate formed through the inter-action of the antigen and antibodies in the system.

The range of pH for all reactants was between 7.0 and 7.2. Repeated precipitin tests were performed with an experimental error that did not exceed ± 2½ per cent.

Plotting of titration curves

Corrected values for antigen and antibody precipitates were plotted with the turbidities on the ordinate and the antigen dilutions plotted on the abscissa. The initial concentration of antigen was always placed at or near the intersection of the ordinate and abscissa. The homologous reaction (the reaction between an antiserum and the antigen used for its production) is the standard of reference for all heterologous reactions (the reaction between the same antiserum and the other antigens).

Values which are proportional to the areas of the curves are obtained by summating the plotted turbidity readings. These numerical values are statistical indices which serve to characterize curves for comparative purposes. The "areas" plus the plots of the curves provide analytical data on the biochemical nature of the hemocyanins which are being compared.

Preservatives in reactants

Marusich (1949) found that "Merthiolate" in the concentrations used in this study (1 : 5,000, and 1 : 10,000) did not alter the serological activity of hemocyanins or the antisera produced against them. Leone (unpublished data) found that formalin at a concentration of 1 : 500 altered the serological activity of hemocyanins slightly but not enough to modify significantly the data which are presented in this paper.

EXPERIMENTAL RESULTS

A summary of the serological relationships based on "areas" which were calculated from turbidity measurements taken at 20 minutes and 24 hours is presented in Table II. Plots of representative precipitin-curves are given in Figures 1 and 2.

TABLE II

Serological relationships among species belonging to the families Astacidae, Parastacidae and Homaridae from turbidity readings at 20 minutes and 24 hours

Antisera	Anti-*Orconectes neglectus* (Astacidae)		Anti-*Orconectes immunis* (Astacidae)		Anti-*Orconectes nais* (Astacidae)		Anti-*Orconectes virilis* (Astacidae)		Anti-*Procambarus simulans* (Astacidae)		Anti-*Cherax albidus* (Parastacidae)		Anti-*Cherax destructor* (Parastacidae)		Anti-*Euastacus nobilis* (Parastacidae)		Anti-*Homarus americanus* (Homaridae)	
Antigens																		
Orconectes neglectus	100	100	81	71	80	73			95	85								
Orconectes immunis	82	83	100	100	94	92	68	69	91	91	28	33	37	34	27	37	69	68
Orconectes nais	93	93	90	77	100	100	95	95	90	93	29	41	31	26	20	33	50	64
Orconectes virilis			73	68	81	81	100	100	93	96	33	37	26	26	23	35	50	64
Procambarus simulans			94	91	74	74			100	100	40	52	24	26	27	41	44	52
Cherax albidus	63	63	32	46	49	55	42	54	18	37	100	100	96	98	53	68	28	28
Cherax destructor	43	43	36	37	24	38	31	50	43	53	97	98	100	100	55	68	60	66
Euastacus elongatus					56	65	43	52	61	71	81	75	80	74			48	61
Euastacus nobilis	29	30	27	26	26	36			41	50	59	58	52	54	100	100	57	63
Euastacus armatus	16	22	30	34	25	35	38	43	44	54	65	69	53	54	90	92	55	64
Homarus americanus	17	21	31	37	26	32	33	38	5	14	11	26	1	6	19	25	100	100

Antisera produced with the small amounts of hemocyanins as given in Table I proved to contain sufficient quantities of precipitins to permit serological comparisons. Heterologous reactions did not in any case exceed homologous reactions. That the heterologous reactions did not exceed homologous reactions is in accord with the researches of Landsteiner (1945, p. 272), who found that antisera contain varying proportions of unique substances which will combine only with the homologous antigens. Heterologous antigens do not possess radicals identical to those of the homologous antigens, but may possess similar substances which allow reactions. Leone (1950, p. 124) and Boyden (1942) have defined this reaction as serological correspondence. Such correspondence when applied to antigens includes all antigens capable of reacting with an antiserum to any one of them, and when applied to antisera includes all antisera capable of reacting with any given antigen.

The precipitin curves proved to have single, narrow peaks, which strongly suggests that a single antigen-antibody system is involved in the reactions. In two instances, however, plots of turbidities revealed curves (Figures 1A and 1B) which were bimodal. Variations from a single modal curve have been considered as evidence of the probability of the presence of more than a single kind of antigen or

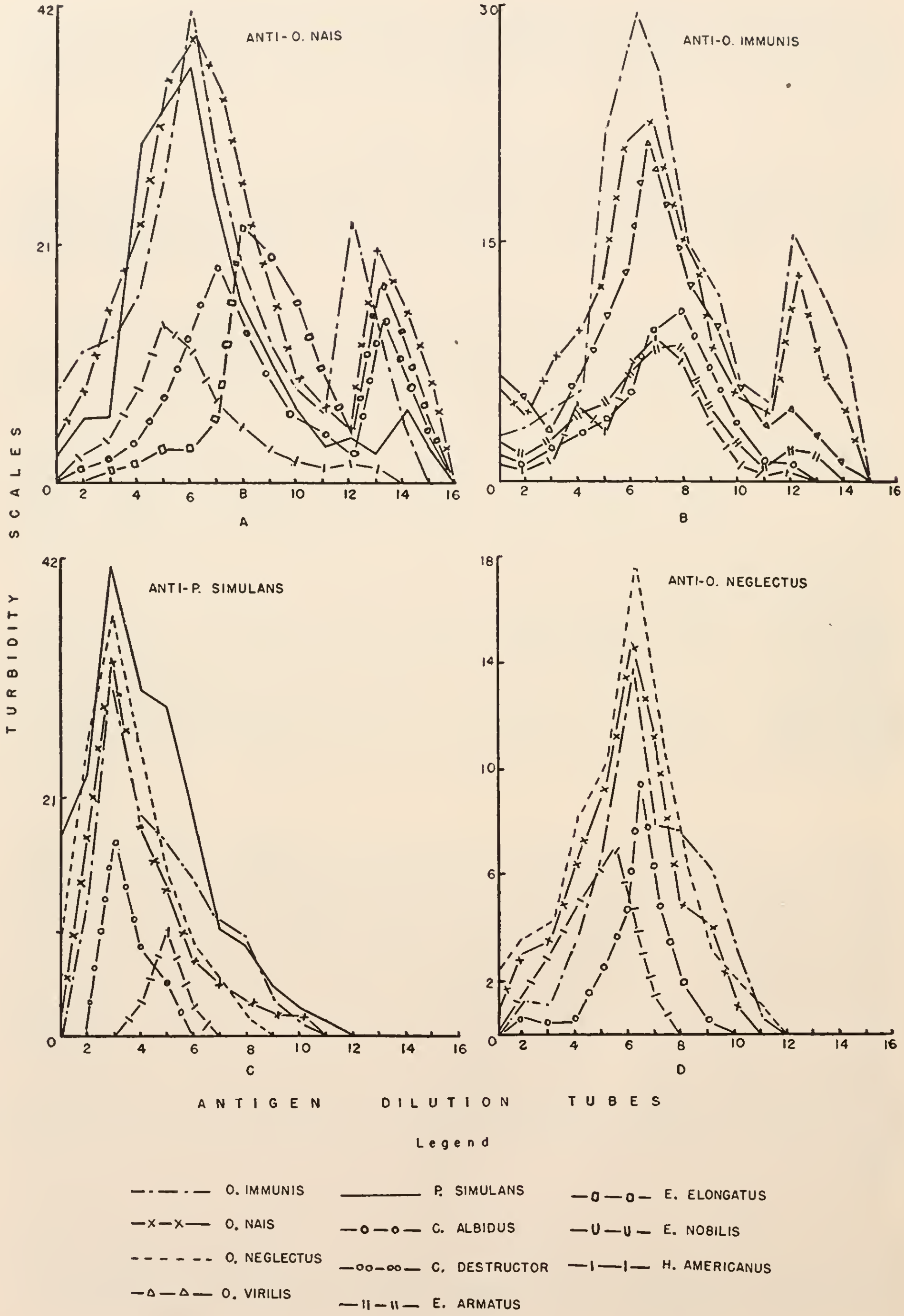
ANTI-O. NAIS
ANTI-O. IMMUNIS
ANTI-P. SIMULANS
ANTI-O. NEGLECTUS
TURBIDITY SCALES
ANTIGEN DILUTION TUBES
A
B
C
D
0
2
4
6
8
10
12
14
16
21
42
15
30
10
14
18
Legend
O. IMMUNIS
O. NAIS
O. NEGLECTUS
O. VIRILIS
P. SIMULANS
C. ALBIDUS
C. DESTRUCTOR
E. ARMATUS
E. ELONGATUS
E. NOBILIS
H. AMERICANUS

FIGURE 1

antibody in the system (Leone, 1949). Redfield (1934) concluded that hemocyanins are proteins composed of a prosthetic group consisting of a complex copper salt, of a sulfur compound, and a polypeptide and he indicated that several similar "units" may comprise the whole hemocyanin molecule. Since Svedberg and Pedersen (1940) by ultracentrifugation and sedimentation have been able to determine that hemocyanins have total molecular weights ranging from 300,000 to several million and are composed of polymeric fractions, it may well be that one or more of the groups of these molecular "units" may be responsible for secondary curves or variations from the unimodality of homologous curves.

The methods used in the collection and preparation of the hemocyanins have been kept as simple as possible; as a result little or no detectable chemical changes have occurred in the samples obtained. It is reasonable then to assume that they are representative biochemical entities for the species compared.

Twenty-minute reaction-time has been used extensively by Leone (1950), Boyden (1943), Leone and Pryor (1952) and others. A precipitin reaction may go to completion within 24 hours at room temperature. The binding of antibodies, as a rule, proceeds rapidly and in several experiments of Landsteiner (1945, p. 248) was found to be accomplished, for the most part, in less than a minute and never more than 15 minutes. This binding of antigen with antibody constitutes the first stage of the precipitin reaction. The formation of precipitates constitutes the second stage of antigen-antibody reactions and is a much slower process than is the combination of antigens with antibodies. The temperature at which the reaction takes place must be kept below that temperature which causes deterioration of the reactants. Increases in amounts of turbidities during a "prolonged" reaction-time have been recorded for this study. A homologous reaction, using anti-*Procambarus simulans* serum was read at 20 minutes, 40 minutes, 60 minutes and 24 hours. Increases in the homologous and heterologous reactions were proportional so that values obtained from turbidities after 20 minutes incubation at 37° C. were the same as those developed after 24 hours at room temperature. A homologous reaction using anti-*Orconectes nais* serum and a heterologous reaction using the protein of *Homarus americanus* were read at 20 minutes and again at 24 hours. These tests show increases in turbidities. At the region of optimal proportions higher peaks were obtained but with little or no shifting of these curves toward either the zones of antigen or antiserum excess.

RELATIONSHIPS

Serological tests involving species within the family Astacidae revealed that species within the genus *Orconectes* closely resemble one another and that the genus *Procambarus* is closely related but distinct from the genus *Orconectes* (Fig.

FIGURE 1. A and B. Bimodal curves from reactions of anti-*Orconectes nais* and anti-*Orconectes immunis* sera and the antigens with which they were tested. Reactions for *Orconectes* and *Procambarus* show high degrees of correspondence. Lesser degrees of correspondence are shown for *Cherax* and *Homarus*. C. Reactions of anti-*Procambarus simulans* serum against hemocyanins of closely related Crustacea belonging to the family Astacidae and other Crustacea belonging to the families Parastacidae and Homaridae. D. Reactions of anti-*Orconectes neglectus* serum with closely related Crustacea belonging to the family Astacidae and with species from the families Parastacidae and Homaridae.

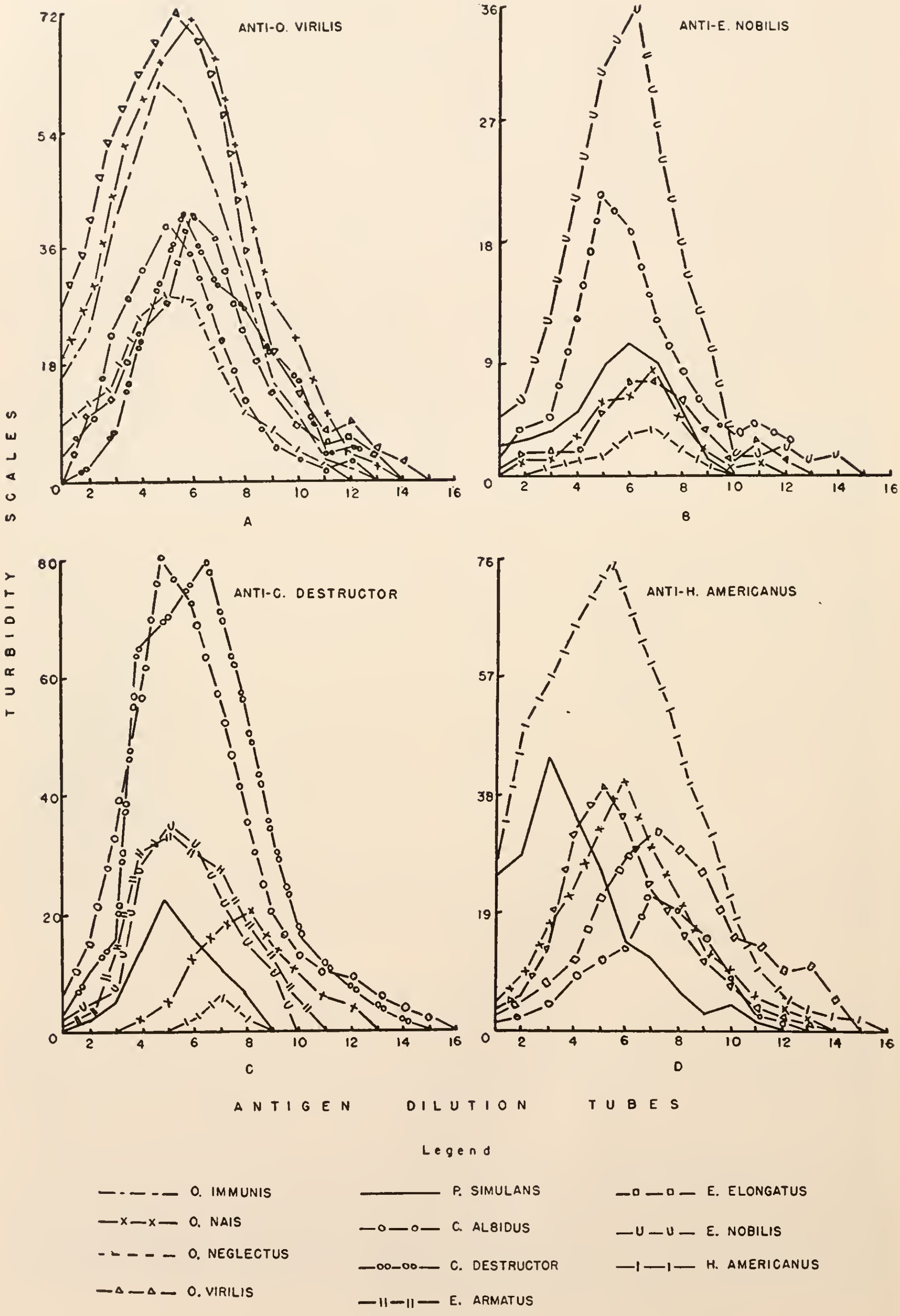
ANTI-O. VIRILIS
ANTI-E. NOBILIS
ANTI-C. DESTRUCTOR
ANTI-H. AMERICANUS
TURBIDITY SCALES
ANTIGEN DILUTION TUBES
72
54
36
18
0
36
27
18
9
80
60
40
20
76
57
38
19
2
4
6
8
10
12
14
16
A
B
C
D
Legend
O. IMMUNIS
O. NAIS
O. NEGLECTUS
O. VIRILIS
P. SIMULANS
C. ALBIDUS
C. DESTRUCTOR
E. ARMATUS
E. ELONGATUS
E. NOBILIS
H. AMERICANUS

FIGURE 2

1, Table II). An exception to expected relationships is indicated by anti-*O. immunis* serum and *P. simulans* protein (Table II). The hemocyanin of *P. simulans* when reacted with anti-*O. immunis* serum exceeds the reactivity of that of species belonging to the genus *Orconectes,* both at 20 minutes and 24 hours. Tests with all other antisera produced against species belonging to the genus *Orconectes* effectively separated *Procambarus* from the genus *Orconectes.* Tests with anti-*P. simulans* serum separated *Procambarus* and *Orconectes.* Some unknown circumstance in laboratory preparation may have altered the serological characteristic of the *Procambarus* proteins. Or, the *Procambarus* hemocyanin actually may possess more of the specific protein radical against which the antibodies were made than do the corresponding proteins of the species of *Orconectes.*

Our interpretation of the data (Table II) is that within the genus *Orconectes,* the species *O. immunis* and *O. nais* are more closely related to each other than to any other of the species tested. *Orconectes virilis* and *O. nais,* which have been considered to be the same species by some authors, appear to be distinct serologically. Both *O. virilis* and *O. neglectus* are more closely related to *O. nais* than to *O. immunis.*

On the basis of tests peformed with anti-Astacidae sera the hemocyanins from species of the families Parastacidae and Homaridae, in every instance, could be separated. The families Astacidae and Parastacidae resemble each other more than either does the family Homaridae. Tests with antisera produced against species of the family Parastacidae likewise separated hemocyanins of this family from those of the family Astacidae (Figs. 2B, 2C, 2D and Table II), and Homaridae. In this instance, also, the Homaridae is the more distant family.

An antiserum produced against the hemocyanins of *E. nobilis* proved effective in distinguishing among proteins of this species and those of species belonging to the families Astacidae and Parastacidae (Fig. 2B). To this antiserum the hemocyanins of species in the genus *Orconectes* and *Procambarus* reacted about equally.

Cherax albidus and *Cherax destructor* are closely related species (Fig. 2C, Table II). Antisera produced to the proteins of the two species closely resemble one another in their specificities. The antiserum against the hemocyanins of *Homarus americanus* gave remarkable differentiation between the two species of *Cherax.* This reaction may be anomalous.

Serological data indicate that *Euastacus armatus* and *E. nobilis* are more closely related than is *Euastacus elongatus* to either of them (Table II). From a study of these proteins, Clark and Burnet (1942) find that *E. armatus* and *E. nobilis* resemble each other more closely than either species does any other of the same genus. The results are also in accord with those obtained by Leone (1950) who found a high degree of serological correspondence among these three species of *Euastacus.*

FIGURE 2. A. Precipitin curves from reactions of anti-Astacidae serum with hemocyanins of species belonging to the families Astacidae, Parastacidae and Homaridae. High degrees of serological correspondence are shown for Parastacidae and Homaridae. B. Anti-Parastacidae serum showing degrees of relationship with hemocyanins from species belonging to the families Astacidae and Homaridae. C. Precipitin-reaction curves showing relationships among members of the families Parastacidae, Astacidae and Homaridae. D. Reactions of anti-*Homarus americanus* serum against hemocyanins of species from the genus *Orconectes, Procambarus, Cherax* and *Euastacus.*

Reactions with the antiserum to *Homarus americanus* indicate that this marine form is serologically distinct from the species in the two aquatic families. Moreover, tests with this antiserum (Fig. 2D, Table II) did not differentiate between Astacidae and Parastacidae. Leone (1950) reacted antisera produced against *Homarus americanus* and *Homarus vulgaris* [= *Homarus gammarus*] with hemocyanins of species belonging to both Astacidae and Parastacidae. He likewise was unable to differentiate these two families from the anti-Homaridae loci.

Discussion

Serological data are of value in constructing a natural system of classification, because (1) proteins are inherited traits which are not subjected to appreciable environmental influence, (2) comparisons can be made between corresponding proteins of the organism, and (3) chemical similarities and differences in antigens are revealed by adequate serological technique.

When corresponding proteins are to be differentiated quantitatively and qualitatively, antigens of known protein concentrations must be compared and the dilutions of each antigen must be equivalent. Antigens must be prepared so that little or no change in the chemical nature of these substances occurs.

In serological testing the rabbit is the greatest variable. Leone (1949, 1950) has shown that different rabbits receiving identical series of injections of the same antigen will produce antisera which differ considerably in their capacities to discriminate among antigens. From this it follows that serological comparisons based upon the production of one antiserum to any given antigen presents the possibility of obtaining only a minimum of data upon which serological comparisons may be based.

Summary and Conclusions

1. Serological comparisons among the hemocyanins of astacuran Crustacea have been determined using the precipitin test and a photoelectric turbidimeter. Five species of crayfish belonging to the family Astacidae (*Orconectes neglectus, Orconectes immunis, Orconectes nais, Orconectes virilis* and *Procambarus simulans*), five species of freshwater Australian crayfish belonging to the family Parastacidae (*Cherax albidus, Cherax destructor, Euastacus elongatus, Euastacus nobilis* and *Euastacus armatus*), and one species of Homaridae (*Homarus americanus*) were compared.

2. The species within the genus *Orconectes* are closely related. Serological data show that *Orconectes immunis* and *Orconectes nais* are most closely related to each other, and that *Orconectes neglectus* and *Orconectes virilis* are more closely related to *Orconectes nais* than to *Orconectes immunis.*

3. *Procambarus simulans* is closely related to species in the genus *Orconectes.*

4. The families Astacidae, Parastacidae and Homaridae are serologically distinct from one another. Tests with antisera produced against either the Astacidae or the Parastacidae have been able effectively to separate the other two families. On the other hand, tests with the anti-homarid serum were unable to separate differentially the families Astacidae and Parastacidae.

5. Within the family Parastacidae the genera *Cherax* and *Euastacus* are notably different serologically, but are more closely related to one another than to species from the Astacidae or Homaridae.

6. *Euastacus armatus* and *Euastacus nobilis* are more closely related to each other than either is to *Euastacus elongatus.*

7. *Homarus americanus,* in the family Homaridae, is distantly related to species belonging to the families Astacidae and Parastacidae.

8. Comparisons of relative relationships at 20-minute and 24-hour reaction times in serological relationships have been made.

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AN ANALYSIS OF THE AGGREGATION STAGE IN THE DEVELOPMENT OF THE SLIME MOLDS, DICTYOSTELIACEAE. II. AGGREGATIVE CENTER FORMATION BY MIXTURES OF DICTYOSTELIUM DISCOIDEUM WILD TYPE AND AGGREGATELESS VARIANTS[1]

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In the development of the Dictyosteliaceae, aggregation begins when the population has reached the stationary growth phase. The individual myxamoebae, previously distributed at random, become elongated and radially oriented. They move in toward the centers of orientation and are crowded together in ramified streams as they do so. Mounds of cells are built up in this fashion and acquire the characteristic form of the pseudoplasmodium. Ultimately, the pseudoplasmodia give rise to organized, multicellular fruiting structures (Bonner, 1944).

Several features of this phenomenon have been investigated. Evidence obtained by Bonner (1947) supports the conclusion that aggregation is the result of a chemotactic response on the part of outlying cells to the production of a diffusible substance by individuals at or near the aggregative center. Study of interspecific and intergeneric cell mixtures (Raper, 1940) has indicated that not all species can enter communal aggregates and suggests that the aggregative response is specific.

The number of aggregative centers that can be formed by the myxamoebae is a function of both the population number and density (Sussman and Noël, 1952). Where the population density does not limit center formation, the number of centers produced is proportional to the number of cells. For *Dictyostelium discoideum* strain Nc-4, the number of centers/cell was found to be 4.73×10^{-4}, corresponding to a distribution of one center-initiating agency among approximately 2100 cells. For *D. purpureum* strain V-1, the value was 3.3×10^{-3}, equivalent to one center-forming agency among 300 cells. Examination of the distribution of center-forming capacity among small, replicate population samples revealed that under the conditions employed only a small proportion of the population possesses the ability to initiate the aggregative process.

One pertinent question, which could not be answered by study of the wild type populations exclusively, is concerned with whether or not single cells act as initiators of aggregation. The reasons for this limitation are given in another part of this paper. In order to settle the question, an analysis has been made of aggregations carried out by mixtures of wild type myxamoebae and aggregateless variants which are capable of responding to the aggregative stimulus but not of producing it. The results demonstrate the single cells do indeed initiate aggregation. Several other aspects of these mixed aggregations have been investigated and are described.

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METHODS

A. Organisms and growth conditions

The strains used in this investigation were: *Dictyostelium discoideum* strain Nc-4 and two aggregateless variants of this stock, Nc-4 agg-53 and Nc-4 agg-53A. These were routinely grown in association with *Aerobacter aerogenes* on an agar medium whose composition has been given elsewhere (Sussman, 1951).

The technique of clonal plating, used in certain of the experiments reported here, has been previously described (Sussman, 1951). It is similar to the "spread plate" technique employed in the past for bacteriophage (Delbrück, 1946). The plaques of myxamoebae appear within the layer of bacterial growth after 3.5 days incubation at 22° C. During the following 24 hours, fruiting structures appear in the plaques.

B. Preparation of cells for aggregation experiments

44- to 50-hour plates containing myxamoebae in the stationary growth phase were harvested with cold, distilled water. The suspensions were spun in an International refrigerated centrifuge for 5 minutes at 1000 rpm in order to separate the myxamoebae from the remaining bacteria. After three more washes, the cells were suspended in Bonner's salt solution (Bonner, 1947). At this time, direct cell counts of the suspensions were made in the Levy chamber on 6 to 8 replicates. The variances encountered were satisfactorily small.

C. Deposition of cells on aggregation plates

The aggregation experiments were carried out on a washed agar-distilled water substratum, hereafter denoted as "minimal agar." This was prepared by washing 25 g. of Difco agar in 6 liters distilled water on a Buchner table-top filter. After washing, the agar was suspended in one liter of water and autoclaved. Plates were poured from this medium.

When aliquots of cell suspensions, ranging in volume from 0.01 to 0.2 cc., are placed on minimal agar, the cells are distributed homogeneously over a surface area whose size is a function of the volume delivered. In this manner, both the population number and density may be controlled effectively and the myxamoebae are found to aggregate and produce normal fruiting bodies.

Experiments to be described indicate that when cells are prepared by the procedure given and are deposited on minimal agar, no appreciable increase in cell number occurs after as much as 48 hours incubation. It must be noted, however, that the separation of myxamoebae from occluded bacteria is occasionally unsatisfactory. With such preparations, no trouble is encountered where the population on the minimal agar plates is very dense since the amoebae ingest the bacteria almost immediately. But in aliquots where the population density is not great, some bacteria escape ingestion long enough to form microcolonies. When these are finally entered by the myxamoebae, substantial proliferation ensues. This difficulty can be avoided almost completely by careful washing of cells and of the agar used in the minimal medium.

RESULTS

A. Descriptions of the aggregateless variants

Two variants were employed in this investigation. The first of these, Nc-4 agg-53, was obtained by irradiation of the wild type with UV. The techniques used for its isolation as well as for the collection of a series of stocks displaying a wide spectrum of aberrant developmental patterns will be described elsewhere (Sussman and Sussman, in preparation).

The plaques produced by agg-53 are easily distinguishable from those of the wild type. The latter forms a spreading plaque with an irregular outline and diffuse periphery. Normal fruiting structures appear within its confines. In contrast, the plaque formed by agg-53 is much smaller, circular and has a very sharply defined periphery. A circular slime deposition or series of concentric depositions always appears, giving each plaque the appearance of a bull's eye. No signs of aggregation or the erection of fruiting structures are observable. Spores are not produced.

During serial passage of agg-53, a plaque type variant was found to arise occasionally. Upon sub-culture, it bred true and was designated Nc-4 agg-53A. The plaque produced by this variant spreads and has an irregular outline like that of the wild type. The interior of the plaque remains perfectly smooth. No signs of aggregation or the erection of fruiting structures are seen. Spores are not formed.

In the course of many serial passages, neither of the variants has been seen to produce a reverted or mosaic clone. However, this extreme stability is by no means true of all aggregateless stocks thus far isolated.

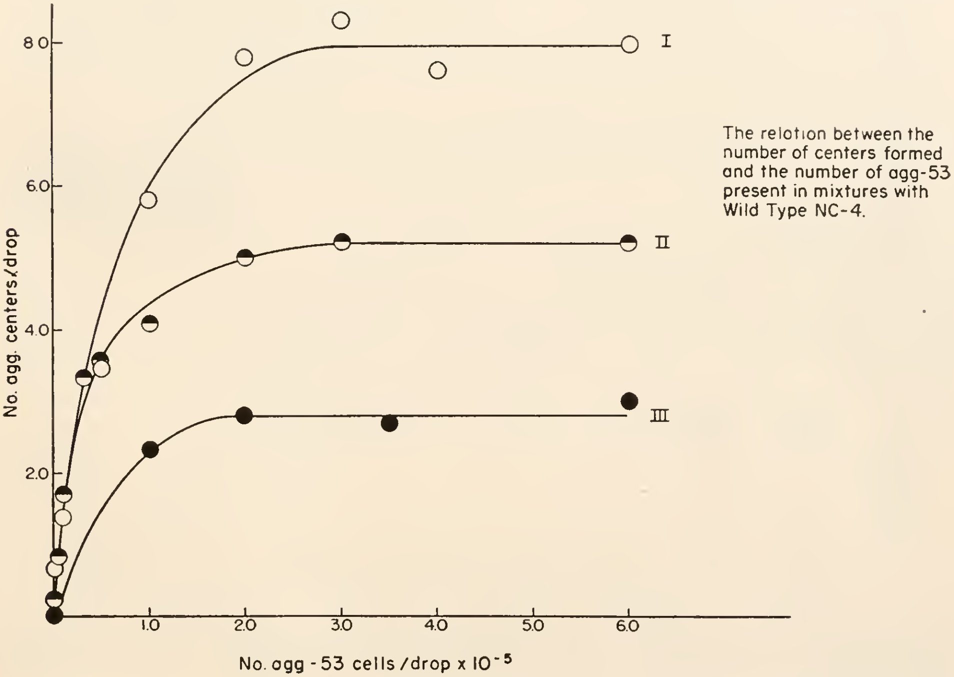
The question of whether or not the strains represent genic modifications cannot be answered since, at present, no system of recombination analysis is available for use. Arguments not based on segregation data are neither necessary nor sufficient for the distinction to be drawn and therefore will not be considered at this time.

It should be noted that Pfützner-Eckert (1950) has reported the isolation of a strain of *D. mucoroides* which is incapable of forming fruiting structures. From this author's description, however, it appears that the cells can perform some of the activities associated with aggregation and, under some conditions, even form small pseudoplasmodia. We have isolated similar varieties. Such cells when washed and distributed on minimal agar generally are capable of aggregating and producing normal mature fruiting structures although usually not in as great a number as the wild type produces. The inability of such cells to do the same on the growth plates is a problem which has not as yet been attacked.

B. The formation of aggregative centers by mixtures of Nc-4 wild type and the variants

A previous investigation (Sussman and Noël, 1952) has demonstrated that the number of aggregative centers produced by Nc-4 wild type cells is a function not only of the number of cells present but also of the population density. When the latter is less than approximately 100 cells/mm.^{2}, no centers are formed. As the density is raised (while the number of individuals is kept constant), the number of centers increases until at a density of about 200 cells/mm.^{2} a maximal number of centers is produced. This circumstance made it possible to determine if the aggregateless individuals could respond to the stimulus imposed by wild type where the population density of the wild type was so low that, alone, they could not form centers.

Suspensions of washed Nc-4 wild type and agg-53, prepared according to the procedure previously described, were counted in the Levy hemocytometer and appropriate dilutions were made. From these, mixtures were arranged such that they contained a constant number of Nc-4 cells but varying numbers of aggregateless individuals. For example, in one experiment the mixtures all contained 5.0×10^5 cells/cc. of wild type while the numbers of agg-53 cells/cc. ranged from 5.0×10^5 to 6.0×10^7. Replicate drops of 0.01 cc. volume were distributed on minimal agar, 10 drops to a plate and two or three plates for each mixture. Thus, the number of Nc-4/drop was 5.0×10^3 while the numbers of agg-53/drop varied between 5×10^3 and 6.0×10^5. Drops were also dispensed from control suspensions containing either Nc-4 or agg-53 in concentrations comparable to those in the experimental suspensions.



The relation between the number of centers formed and the number of agg-53 present in mixtures with Wild Type NC-4.

FIGURE 1. Curve I refers to mixtures containing 8.0×10^3 wild type cells/sample with various numbers of agg-53 cells. Curve II refers to mixtures containing 5.0×10^3 wild type/sample and Curve III, 2.6×10^3.

After distribution of the drops, the plates were allowed to stand with covers ajar until the drops had dried. The plates were incubated at 22° C. and twice during the following 48 hours the drops were examined either under 20 × or 100 × magnification in order to count the aggregates or fruiting bodies derived therefrom.

Thirteen of these determinations were made using seven different quantities of Nc-4 cells/drop ranging from 1.0×10^3 to 1.0×10^4. Figure 1 shows the results of three such experiments. Each point in the curves represents the mean of 30 replicate drops.

In the absence of agg-53 cells, 2.6×10^3 Nc-4 produced no centers (curve III of Fig. 1), as a result of the extremely low population density. (From the average radius of the drops and the number of cells deposited, the density was estimated to be less than 50 cells/mm.^{2}) Alone, 5.0×10^3 Nc-4 formed a mean of 0.21 centers/drop; 8.0×10^3 Nc-4 produced 0.7 centers/drop. Control suspensions of agg-53 cells were never observed to give rise to aggregate centers. Addition of agg-53 cells to the wild type suspensions, however, markedly increased the numbers of centers formed. The presence of between 2 and 3×10^5 agg-53 cells resulted in maximal center formation whose absolute value depended upon the number of wild type. Thus for 8×10^3 Nc-4, a plateau value of 7.9 centers/drop was reached. For 5×10^3 Nc-4 the value was 4.9 and for 2.6×10^3 Nc-4 it was 2.8. One center was produced for approximately 1000 wild type cells present.

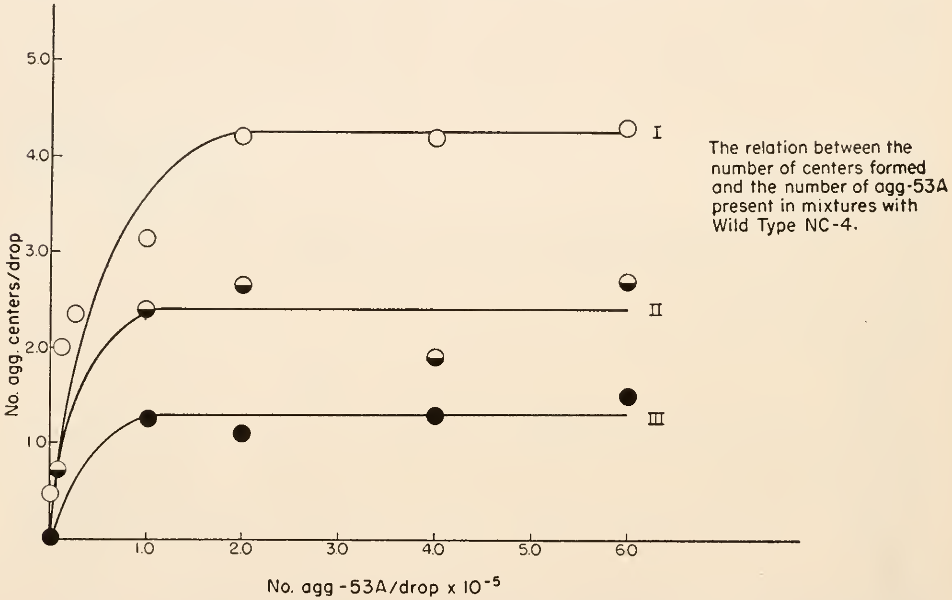


FIGURE 2. Curve I refers to mixtures containing 8.0×10^3 wild type cells/sample with various numbers of agg-53A. Curve II refers to mixtures containing 5.0×10^3 wild type cells/sample and Curve III, 2.0×10^3.

A similar set of experiments was performed using mixtures of Nc-4 and agg-53A. Figure 2 summarizes the results of three determinations. The curves are in qualitative agreement with those of Figure 1 except that significantly different plateaus were reached for the same numbers of Nc-4 employed. Using agg-53A as the test system, one center was formed for approximately every 1700 Nc-4 added.

C. The number of cells required to initiate the formation of an aggregative center

It has been mentioned that the results of a previous investigation indicate that only a small proportion of the myxamoeboid population possesses the ability to initiate center formation under the conditions of the experiment. The question of how many cells were required to initiate a center could not be answered conclusively from the data although a number of arguments could be raised to support the interpretation that a single cell can evoke the aggregative process. The inconclusiveness proceeded from the fact that in a population of wild type cells, one is concerned not only with the ability of a small proportion to initiate aggregation but also with the ability of the remaining individuals to respond to the initiating stimulus. From examination of the dependence of center formation on population number and density, it became clear that both abilities played decisive roles in the process. Thus, one could not, with this system, hope to dissociate the one from the other.

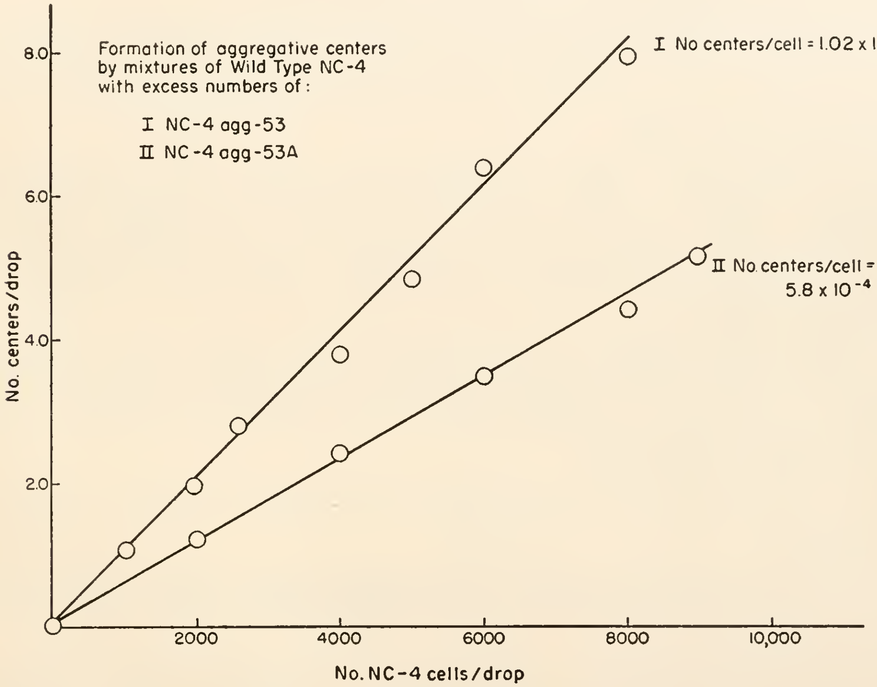


FIGURE 3. See text for experimental details.

The use of aggregateless variants as the test system was considered to be capable of achieving the desired dissociation since with this system it became possible to alter the number of responding cells to any extent without changing the number of wild type individuals and therefore the number of initiator cells. Conversely, the presence of excess aggregateless cells would ensure that neither the number nor density of responding cells would limit the formation of centers. In this event, alteration of the number of wild type cells could only affect the number of initiators and it became possible therefore to examine initiating ability, *per se*.

Where the responding cells could not limit center formation, the initiation of centers by single cells would be evidenced by the fact that the number of centers formed would be directly proportional to the number of initiator cells present. For mixtures of Nc-4 and excess aggregateless variants, this would mean that the number of centers would, in fact, be proportional to the number of wild type, *i.e.,* the ratio of number of centers/wild type cell would remain constant regardless of the absolute number of wild type present. If, however, two or more cells must collaborate to evoke aggregation, the number of centers would in general vary exponentially with the number of wild type present and the ratio, number of centers/wild type cell, would approach the limit zero at infinite dilution.

A distinction between these possibilities could be made by plotting the plateau values obtained from the experiments described in section B against the numbers of Nc-4 present. It will be recalled that the plateau values represent the number of centers formed by Nc-4 initiators in the presence of excess aggregateless cells. Figure 3 shows the results.

Curve I of Figure 3 was obtained for mixtures of Nc-4 and agg-53, curve II for Nc-4 and agg-53A. Both are straight lines, indicating that with either test system, the number of centers formed is proportional to the number of wild type present, *i.e.,* the ratio of centers/wild type cell remains constant regardless of dilution. It must be concluded that for *D. discoideum,* at least, single cells do act as aggregation initiators.

In mixtures with agg-53 the mean of the centers/Nc-4 cell was calculated to be 1.02×10^{-3}, which corresponds to a distribution of one initiator cell per 980 individuals. For mixtures with agg-53A the mean was 5.8×10^{-4}, equivalent to a distribution of one initiator per 1720 individuals. The significance of this discrepancy will be discussed subsequently.

D. Proof that aggregateless cells enter into the aggregations evoked by wild type initiators

The fact that mixtures of Nc-4 and the aggregateless variants form centers whereas neither can do so alone under the conditions of test may be explained by at least four suppositions.

(a) The entire population proliferates on the minimal agar plates. Each of the cells originally laid down therefore produces a microclone. The Nc-4 cells increase in number to the extent that some can aggregate without assistance from the aggregateless individuals.

(b) The total population remains relatively constant on the minimal agar. However, the wild type cells increase in number at the expense of the aggregateless individuals. This increase might be due to growth or conceivably to the transformation of aggregateless variants into wild type. In either case the proportion of Nc-4 in the population would rise. The net increase would be sufficient for them to aggregate without assistance from the agg-53 cells.

(c) The numbers of both Nc-4 and agg-53 cells remain constant. The aggregateless cells act as "conductors" of the stimulus imposed by initiator cells in the wild type population. This conductance enables the responding cells of the wild type population to aggregate without, however, the active participation of the aggregateless individuals in the aggregation.

(d) The aggregateless cells, like the responding cells of the wild type population, are affected by the initiating stimulus and enter into the aggregate eventually formed.

It was possible to test the validity of the first two suppositions by making differential and total counts of the mixed population on the minimal agar plates at intervals between the time of deposition and the onset of aggregation. Were assumption (a) valid, the total population would increase significantly. Were assumption (b) valid, the proportion of Nc-4 in the population would increase significantly.

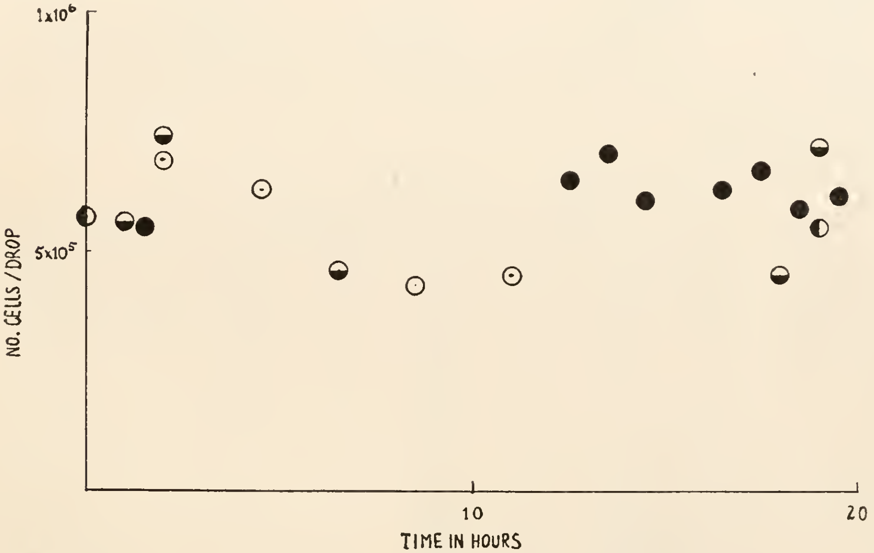


FIGURE 4. Total viable population counts of cell mixtures between the time of deposition and the onset of aggregation. See text for details.

The deposition of drops containing mixtures of Nc-4 and agg-53 on minimal agar plates was accomplished in the usual manner. Two mixtures were used: 10% Nc-4 cells (I) and 1.5% Nc-4 (II). The latter value falls within the limits employed in the previous experiments. The drops were allowed to dry and the plates were incubated at 22° C.

To obtain quantitative recovery of the cells, a flamed cork borer was used to cut plugs in the agar, each plug encompassing the area of a drop. Each plug was then suspended in 5.0 cc. salt solution and thoroughly triturated. Control experiments indicated that between 90 and 100% of the cells may be recovered from the plugs in this manner. Direct counts were made on each of the suspensions and appropriate dilutions were plated in quadruplicate with *A. aerogenes* according to the procedure given in the Methods section for clonally isolated platings. After incubation, total and differential plaque counts were made.

The plugs were collected at intervals until the beginning of aggregation. The drops prepared from mixture I showed evidence of aggregation at 12–14 hours. The cells in mixture II aggregated at 20–23 hours. Samples could not be taken after these times because of excessive clumping of the cells and adherence to the agar.

Figure 4 illustrates the total population counts from four experiments. It will be noted that the viable counts cover the entire span between the deposition of the cells and the onset of aggregation. Each point represents the mean of counts obtained from four to eight plugs (16 to 32 plates). It is clear that no significant increase in cell number occurred on the aggregation plates. The mean number of plaques/plate was 59.2 with a variance of 109. The direct cell counts remained constant as well. The fact that extensive proliferation does not occur makes supposition (a) untenable.

Table I summarizes the results obtained from differential plaque counts. Neither mixture I nor II showed a significant change in the proportion of Nc-4 cells between zero time and the onset of aggregation. It must be concluded that assumption (b) is invalid.

TABLE I

The proportion of wild type cells in the mixed populations during incubation on minimal agar

Mixture	Time in hours after deposition	Total no. plaques	No. wild type plaques	% Wild type
I	2	1103	101	9.2
	4.5	1198	93	7.8
	8.5	347	31	9.0
	11	665	54	8.1
II	1–2	1019	15	1.5
	4.5	1096	16	1.5
	8.5	340	5	1.5
	11 –13.5	949	16	1.7
	14.5–16.5	501	10	2.0
	17.5–23.5	2095	34	1.6

Replicate population samples were collected at the stated intervals, suspended and plated. After incubation the plaques were surveyed and the proportions of wild type clones were determined.

Microscopic observation of aggregations of mixed populations has made supposition (c) highly unlikely. When the number of aggregateless cells is not overwhelmingly large, it may be seen that the entire area immediately surrounding the completed aggregate has been cleared of cells. Were the aggregateless individuals serving merely as conductors of the stimulus and not entering the aggregates, it would be impossible for every cell near the aggregate to have been removed since the ratio of aggregateless cells to wild type in such mixtures can be as high as 50:1. Moreover, visual comparison of aggregative patterns produced by control suspensions of the wild type alone and in conjunction with aggregateless cells revealed that the latter were far more extensive than the former.

In the absence of any other interpretations, one must conclude that the aggregateless cells do indeed enter into the aggregations evoked by wild type initiators. In this connection, the results of Pfützner-Eckert (1950) are of interest. Myxamoebae of the strain incapable of fruiting were shown to orient around and stream toward wild type pseudoplasmodia which had been placed in their midst.

E. The phenotypic composition of the sori from mixtures of wild type and agg-53

Mixtures of wild type and agg-53 were prepared in the usual manner and dispensed on minimal agar. The following aliquot volumes and proportions were employed:

Mixture	Vol. of aliquots delivered	No. Nc-4	No. Agg-53	Ratio Nc-4:Agg-53
1	0.01 cc.	6.0×10^3	3.0×10^5	1:50
2	0.02 cc.	6.0×10^3	5.0×10^5	1:83
3	0.04 cc.	6.0×10^3	8.4×10^5	1:139
4	0.08 cc.	6.0×10^3	1.4×10^6	1:232

In this manner a constant number of wild type cells was distributed over different surface areas while the variation in numbers of agg-53 provided constant population density. The particular concentrations of agg-53 were chosen on the basis of experiments (Sussman and Noël, 1952) from which the relation between the volume of aliquot and the surface area covered had been determined.

After the plates had been incubated for 36 hours, approximately 6 aggregative centers/aliquot had been formed in each of the mixtures. Single spore masses were picked and plated with bacteria. After four days incubation, the plaques were examined. The approximate numbers of plaques surveyed for the four mixtures cited above were, respectively, 1430, 1000, 350 and 204. In every case, not a single agg-53 plaque was observed. All produced normal fruiting bodies.

These results would appear to indicate that aggregateless cells do not enter into the aggregates initiated by the wild type. However, in view of the results described in the previous section, this is not considered to be a likely possibility. Two additional explanations can be offered. First, it may be presumed that only the wild type cells within the mixed aggregates eventually become spores. Second, one might suppose that any aggregateless cells which enter the aggregates either spontaneously revert or are transformed to wild type. The implications which the latter possibility possesses both for the genetic nature of the aggregateless variant and for cellular interactions during the developmental process would seem to demand that a distinction between these interpretations be attempted.

DISCUSSION

The information at present available makes it possible to formulate a description, albeit tentative and incomplete, of the myxamoeboid population undergoing aggregation. During the stationary growth phase, the wild type population may be divided into at least two phenotypic classes. These are the initiator cells, each of which can evoke the formation of an aggregative center, and the responding cells, which can proceed to aggregate only upon reception of the stimulus imposed by an initiator cell.

Two independently derived sets of data indicate that only a small proportion of the cells possesses initiating capacity under the conditions of test. The precise proportion of initiators in *D. discoideum* is in doubt. An examination of center formation by individuals of strain Nc-4 had previously indicated that there exists one initiator cell per 2110 myxamoebae (Sussman and Noël, 1952). But the results obtained from mixtures of wild type with Nc-4 agg-53A indicate a ratio of one initiator per 1720 cells and a corresponding analysis employing Nc-4 agg-53 as the test system suggests a value of one initiator per 980 cells. The discrepancy between the first two values is probably significant, that between the first or second and the last is certainly so.

A tentative explanation of these differences, which we favor currently as a working hypothesis, rests upon two assumptions: (a) There is a distribution of initiating ability in the wild type population, a few cells possessing a very high order, others inferior orders. These inequalities may stem from differences in a particular biosynthetic capacity which is associated with the process of initiation. (b) The ability of cells to respond to the initiating stimulus is a function of experimental conditions such as population density and depends also upon the strain of organisms employed.

From these suppositions, one might expect that for responding cells which possess a low level of sensitivity, only a portion of the potential initiators might have acquired sufficient initiating capacity to evoke the aggregative process. The greater the sensitivity of the responding cells, the higher would be the proportion of individuals which could be classed as initiators. In fact, were responding cells available which possessed extreme sensitivity, quite possibly the entire wild type population might be shown to have attained some degree of initiating ability.

On this basis, the previously cited discrepancies become understandable. When wild type individuals are mixed with aggregateless variants, the proportion of initiators will depend upon the sensitivity of the particular variant employed, hence the difference in the results obtained with agg-53 and agg-53A. Given aggregateless cells whose sensitivity approaches that of the responding cells in the wild type population, the proportion of initiators which appears in the mixture would be higher than when the wild type itself serves as the test system, even when the latter is at optimal population density. This stems from the fact that the density of responding cells in the former case can be made enormous and consequently even relatively small initiating capacities of some wild type cells would evoke a response. When wild type is employed alone, however, this concentration of responding cells cannot be attained. If one were to move the responding cells closer together, one would be simultaneously concentrating the initiator cells. The closer the proximity of these cells, the greater would be the opportunity for them to compete for responding cells, with the inevitable result that the maximal number of centers of which the population is capable would not appear and the apparent proportion of initiators would be decreased. Experimental evidence (Sussman and Noël, 1952) indicates that at population densities greater than 200–300 cells/mm.^{2} the decrease becomes significant and progressively larger. But this density is two orders of magnitude below that which can be achieved by addition of aggregateless cells.

While this is by no means the only interpretation which can be drawn, it offers readily testable inferences that are under examination at present. Its value lies in the fact that it offers an explanation of the genetic mechanism controlling the observed expression of cell heterogeneity. It suggests that the differences between initiator and responding cells may not be genic in nature but rather expressions of enzymatic adaptation against a constant genic background. That phenotypic alterations involving relatively small segments of a population can be ascribed to non-genic variations in enzyme-forming capacity has been demonstrated in connection with the phenomenon of long-term adaptation in yeast (Spiegelman, Sussman and Pinska, 1950).

It is a pleasure to acknowledge the valuable assistance of Mrs. Min Zakarian in the performance of the experiments reported here.

SUMMARY

1. A study has been made of the formation of aggregative centers by mixed populations of *D. discoideum* wild type and aggregateless variants. The latter are capable of responding to the aggregative stimulus but not of producing it. The following results were obtained:

(a) Under conditions where the wild type is too dispersed to aggregate alone, addition of aggregateless cells permits the formation of aggregative centers. When the number of centers so formed is plotted against the number of variant cells present, a saturation curve is obtained.
(b) For the case where the aggregateless cells are present in excess, the number of centers formed is directly proportional to the number of wild type added.
(c) Neither the total population nor the proportion of wild type cells changes significantly during the period of incubation prior to the onset of aggregation.

2. These findings demonstrate that the evocation of an aggregative center is accomplished by a single individual in the wild type population. These have been termed "initiator" cells. Under the conditions employed, the remainder of the wild type individuals and the aggregateless variants are the "responding cells" which can aggregate only in the presence of an initiator.

3. Estimates made of the proportion of initiators in the wild type population, using as test systems the wild type population itself as well as two aggregateless variants, range between one per 980 and 2110 individuals. The discrepancy is considered to be real and an explanation is offered.

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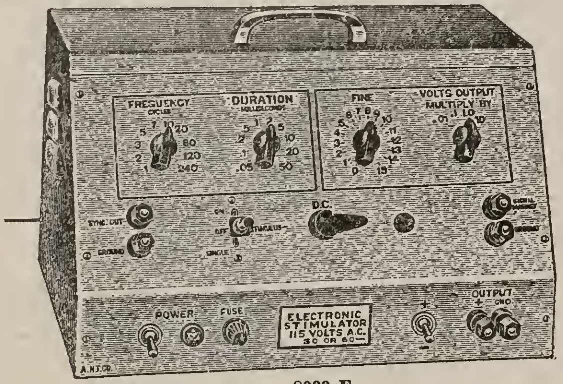

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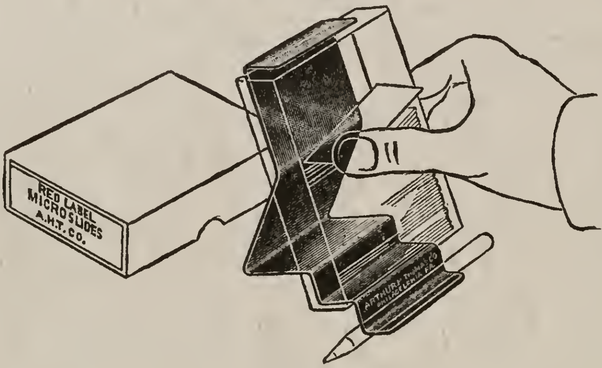
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